IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	LaVallie et al.		
Application No.:	08/949,904	Group Art No.:	1642
Filed:	October 15, 1997	Examiner:	Ungar
For:	Human SDF-5 Protein and Composition		
Confirmation No.:	8744		
Customer No.:	22204		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration Under 37 C.F.R. §1.132

Sir:

I, Edward R. LaVallie, declare the following in support of the above-identified application.

I am a Ph.D. candidate in the Department of Pharmacology at Boston University School of Medicine, nearly completed with the dissertation research. I hold an M.S. from the University of Connecticut in Microbiology. I also hold a B.S. in Biology from American International College.

I currently serve as a Senior Scientist /Associate Director at Wyeth where I work on research and development on osteoarthritis, muscular dystrophy, atherosclerosis, obesity, and asthma in the Department of Discovery Medicine. I have previously worked on hematopoeisis and the bacterial expression of mammalian proteins. I have worked continuously in the biotechnology and pharmaceutical industries for over twenty-one years.

I have included an updated copy of my *curriculum vitae* (Exhibit A) documenting my educational and professional background, along with a list of publications, patents, and presentations on which I have contributed as an author.

By virtue of my research, I am very knowledgeable regarding the current literature, theory, and recent developments relating to *in vitro* and *in vivo* models.

I am familiar with U.S. Patents, and I am named as an inventor on many pending patent applications and on thirty-seven (37) issued U.S. patents.

I am submitting this Declaration on behalf of the Assignee of the instant application in order to declare that the *in vitro* data in Example 7 of the specification (pgs. 52-53) represents an acceptable *in vitro* model that is expected to reasonably correlate with *in vivo* results. The *in vitro* data is derived from the MLB1.2MYC-clone 14 cell line, as described in Rosen, V., et

al. (*Journal of Bone and Mineral Research*, 9(11): 1759-1768 (1994)) hereinafter "Rosen" (Exhibit B).

I am familiar with the prosecution history of this patent application, having read in particular the specification, the currently pending claims, and the Examiner's position regarding *in vitro* data, as set forth in the Office Action dated November 12, 2003 (Exhibit C).

In order to analyze the disclosed *in vitro* utility, I reviewed Rosen in light of my own knowledge of the state of the art relating to cell lines and *in vivo* activity. Specifically, I reviewed the specification and Rosen in order to determine if the activity of the cell line should reasonably correlate with *in vivo* activity.

The Examiner alleges that with regard to "the *in vitro*, cell culture assay, for the reasons of record, no one of skill in the art would believe that the invention could be used as suggested based only on the cell culture information provided in the specification" (Exhibit C, p. 3, lines 10-13).

Rosen asserts that the response of these cells to growth factors (BMP-2 in the specific example in the paper) is likely to recapitulate the *in vivo* condition; Figure 5 depicts a model of *in vivo* differentiation that the authors propose, based upon their observations with the MLB13MYC-clone 14 cells. Therefore, the authors believe (and we concur) that the *in vitro* responsiveness of these cells can reasonably mimic the *in vivo* condition; in fact, it is the very premise of the paper. The final paragraph in the Rosen paper reiterates this premise.

The increase in cartilage markers in Example 7 demonstrates that SDF-5, in combination with BMP-2, is involved in the regulatory pathway for the formation of cartilage. The present invention, as set forth in the claims, can therefore be used in the treatment of cartilage disorders, such as osteoarthritis, rheumatoid arthritis, and articular cartilage defects. The use of SDF-5 in combination with BMP-2 to increase cartilage formation is a credible utility.

As one of skill in the art, I believe that the *in vitro* data as set forth in the instant patent specification reasonably supports applications *in vivo*. Accordingly, I disagree with the Examiner's assessment set forth in the Office Action dated November 12, 2003, and, in particular, to the statements alleged therein.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Edward R. LaVallie
Senior Scientist
Associate Director
Wyeth Research

NOTARY

Commonwealth of Massachusetts }
ss:
Middlesex }

On the 30th day of March 2004, Dr. Edward R. LaVallie personally appeared before me, known by me to be the same person described in and who executed the foregoing instrument, and acknowledged that he executed the same, of his own free will and for the purposes set forth.

Paula M. Gaete, Notary Public
Commonwealth of Massachusetts
My Commission Expires 10/31/2008

Curriculum Vitae

Edward R. LaVallie

Home:
113 Ann Lee Rd.
Harvard, MA 01451
(978) 772-0355
elavallie@charter.net

Work:
200 CambridgePark Dr.
Cambridge, MA 02140
(617) 665-5029
elavallie@wyeth.com

Professional Experience

Wyeth Research, Cambridge, Massachusetts

3/2003-Present: Senior Scientist/Associate Director, Preclinical Biology Group, Department of Discovery Medicine
Newly formed department within Discovery Research focused on discovery and validation of biomarkers to support Development stage compounds entering clinical trials; testing candidate compounds in *in vitro, in vivo,* and *ex vivo* assays to assist in lead compound selection; animal model validation studies comparing human disease samples to various animal models of human disease; and discovery and validation of novel disease targets in osteoarthritis. I direct a group of 6 scientists with expertise in molecular biology, transcriptional profiling, assay development, and heterologous gene expression, with a focus on osteoarthritis, atherosclerosis and thrombosis, asthma, and musculoskeletal disorders.

1/2002-3/2003: Senior Scientist/Associate Director, Experimental Biology and Target Validation Group, Department of Molecular Medicine / Genomics
Directed a group devoted to identification and validation of novel disease targets discovered primarily through pharmacogenomic methods. My group focused on clinical disease samples and animal models of disease to identify disease-associated genes, and validated these genes through *in vitro, in vivo,* and *ex vivo* assays that we developed and performed. Research projects were directed to osteoarthritis, cystic fibrosis, asthma, and cancer. Advanced novel candidate target genes from our studies into high-throughput screening for new drug discovery in osteoarthritis.

1/2000 – 1/2002: Senior Scientist/Group Head 1
Lead a functional genomics group with responsibility for investigating the function of genes that resulted from a variety of sources, including our own novel gene discovery efforts as well as disease-related genes that were identified in pharmacogenomics efforts ongoing within GI/Wyeth. My group contributed target identification and validation efforts in projects devoted to asthma and cystic fibrosis, osteoarthritis, and Alzheimer's Disease.

Genetics Institute, Inc., Cambridge, Massachusetts

6/97-12/99: Principal Scientist/Group Head 1
Responsible for a group of scientists involved in discovering novel secreted proteins and determining their function. We have accomplished these goals by applying microbiology, molecular and cell biology, protein expression, and in vitro and in vivo assay development to the study of these genes. We applied the Signal Trap technology on non-human (and more recently, human) RNA sources in collaboration with several academic labs to discover therapeutically important proteins. We "mined" the DiscoverEase clone set using bioinformatics, expression profiling using Northerns, in situ hybridization, and Affymetrix gene chips to try to determine function of unknown genes. Several potentially important proteins have been identified and characterized through this effort. Supervised a group of 19 people, including the core DNA Sequencing lab and the core Oligonucleotide and Peptide Synthesis lab, in addition to scientists working on protein expression in E. coli and mammalian cells.

12/95-6/97: Principal Scientist/Group Head 1
Assumed responsibility for converting the "Signal Sequence Trap" technology into a spin-off group called DiscoverEase. Assembled and supervised a large group (>30 scientists) to run the "Signal Trap" yeast selection, perform DNA sequencing, construct cDNA libraries, and isolate full-length cDNA's. Developed an efficient process for cloning, sequencing, performing database searches, and expressing genes encoding novel secreted proteins. Our efforts resulted in the discovery and patenting of several hundred novel secreted proteins. Stayed with the project until it became a separate business unit, when I then moved back into "mainstream" GI Discovery Research.

11/93-12/95: Principal Scientist
Developed and utilized techniques for expression of functional ligand-binding proteins (e.g. cell-surface receptors) in *E. coli*, and applyed this technology to the study of structure/function relationships between bone morphogenetic proteins and their receptors. Also involved in the development and utilization of a novel cloning methodology designed to efficiently and rapidly isolate novel cDNA clones encoding secreted proteins (Signal Sequence Trap) for subsequent characterization and analysis.

11/91-11/93: Staff Scientist II
Cloned a cDNA encoding the catalytic subunit of bovine enterokinase for use as a universal cleaving reagent for thioredoxin fusions. Developed expression systems for production of active enterokinase in both mammalian cells and in *E. coli*. Characterized the activity of the recombinant enzyme, and participated in optimization and scale-up of enzyme production in both systems.

11/89-11/91: Staff Scientist I
Discovered the utility of thioredoxin and thioredoxin-like proteins as fusion partners for the soluble production of heterologous proteins in *E. coli*. Developed expression systems to exploit the utility of this approach, and used the system to produce soluble and

biologically active human Interleukin -11. Devised enzymatic and chemical cleavage methods to allow separation of the component proteins. Designed and performed a purification process for isolation of pre-clinical grade, active and endotoxin-free IL-11. Transferred the resulting process to Process Development for scale-up and ultimately clinical manufacturing.

11/86-11/89 Scientist
Developed and implemented *E. coli* expression systems for the production of homologous and heterologous proteins. Responsible for the expression and production of IL-3, "2nd Generation" t-PA, FGF-4, BMP 5,6 and 7, HIV protease, and protein kinase C. In addition, I developed strains which overproduce methionine aminopeptidase, which is responsible for N-terminal processing of intracellular proteins in *E.coli.*

8/85-11/86 Associate Scientist
Studied flagellin transport in *Bacillus subtilis* with the intention of utilizing this unique export pathway for the excretion of heterologous proteins from this host. Used the cloned flagellin structural gene to create genomic deletion mutants, and studied complemetation of these mutants with various regions of the cloned structural gene. Developed vectors for fusing flagellin to a mammalian growth factor to study expression and transport of a heterologous fusion protein using the flagellin excretion pathway.

8/83-8/85 Assistant Scientist
Developed *B. subtilis* as a host for expression and secretion of human therapeutic proteins. Constructed an expression and secretion system utilizing the promoter and signal sequence from the alpha-amylase gene of *Bacillus licheniformis*, and adapted it for heterologous gene expression *in B. subtilis.*

8/82-8/83 Research Associate
Developed expression systems for overproduction of *B. licheniformis* alpha-amylase in *B. subtilis* in collaboration with Pfizer Central Research. Project involved cloning and amplification of the gene encoding the enzyme in suitable strains to maximize accumulation of the product.

Baystate Medical Center, Dept. of Cardiac Surgery, Springfield, Massachusetts
University of Connecticut Health Center, Farmington, Connecticut
Dr. Richard Engelman, M.D.

6/80-9/81 Research Assistant
Assisted two cardiac surgeons in their clinical research involving optimization of cardioplegia solutions used during open-heart surgery. Collected samples during surgery on human patients, obtained consent forms, and collected and analyzed data. Worked in conjunction with the animal physiology lab at UConn Farmington, which worked with porcine models.

Educational Experience

Boston University School of Medicine, Boston, MA
Ph.D. candidate in Department of Pharmacology and Experimental Therapeutics, Graduate Medical Sciences Division. September 2000 to present. Dr. Andrew Dorner, Ph.D., advisor.

University of Connecticut, Storrs, CT. Master of Science Degree in Microbiology, June 1982. Lawrence Hightower, Ph. D., advisor.

American International College, Springfield, MA. Bachelor of Arts Degree (cum laude) with major emphasis in Biology, minor in Chemistry, May 1980. Recipient of Biology Department Award for excellence in Biology, May 1980.

Publications

1. Donahue, R.E., Seehra, J., Metzger, M., Lefebvre, D., Rock, B., Carbone, S., Nathan, D.G., Garnick, M., Sehgal, P.K., Laston, D., **LaVallie, E.**, McCoy, J., Schendel, P.F., Norton, C., Turner, K., Yang, Y-C., and Clark, S.C. 1988. Human IL-3 and GM-CSF act synergistically in stimulating hematopoiesis in primates. Science 241:1820-1823.

2. **LaVallie,E.R.**, and M.L.Stahl. 1989. Cloning of the flagellin gene from Bacillus subtilis and complementation studies of an in vitro-derived deletion mutation. J. Bacteriol. 171 (6), 3085-3094.

3. **LaVallie, E.R.**, DiBlasio, E.A., Kovacic, S., Grant, K.L., Schendel, P.F., and McCoy, J.M. 1993. A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. Bio/Technology 11, 187-193.

4. **LaVallie, E.R.,** Rehemtulla, A., Racie, L.A., DiBlasio, E.A., Ferenz, C., Grant, K.L., Light, A., and McCoy, J.M. 1993. Cloning and functional expression of a cDNA encoding the catalytic subunit of bovine enterokinase. J. Biol. Chem. 268, 23311-23317.

5. McCoy, J. M. and **LaVallie, E.R.** 1993. Expression and purification of thioredoxin fusion proteins, Unit 16.8. In: Current Protocols in Molecular Biology. Brent, R. and Struhl, K. (Eds.) Greene and Wiley-Interscience, CT.

6. **LaVallie, E.R.,** and McCoy, J.M. 1993. Enzymatic and chemical cleavage of fusion proteins, Unit 16.9. In: Current Protocols in Molecular Biology. Brent, R. and Struhl, K. (Eds.) Greene and Wiley-Interscience, CT.

7. Lu, Z., Murray, K.S., Van Cleave, V., **LaVallie, E. R.,** Stahl, M. L., and McCoy, J.M. 1994. Expression of thioredoxin random peptide libraries on the Escherichia coli cell surface as functional fusions to flagellin: a system designed for exploring protein-protein interactions. Bio/Technology 13, 366-372.

8. Collins-Racie, L. A., McColgan, J. M., Grant, K. L., DiBlasio-Smith, E. A., McCoy, J. M., and **LaVallie, E. R.** 1995. Production of recombinant bovine enterokinase catalytic subunit in Escherichia coli using the novel secretory fusion partner DsbA. Bio/Technology 13, 982-987.

9. **LaVallie, E. R.** 1995. Production of recombinant proteins in Escherichia coli, Unit 5.1. In: Current Protocols in Protein Science. Wingfield, P. (Ed.) Greene and Wiley-Interscience, CT.

10. **LaVallie, E. R.** and McCoy, J. M. 1995. Gene fusion expression systems in Escherichia coli. Curr. Opinion in Biotechnol. 6, 501-506.

11. Lockhart, E., Griffin, J. F. T., Chinn, N., **LaVallie, E. R.,** and Buchan, G. 1996. The cloning, expression and purification of cervine interleukin-2. Cytokine 8, 603-12.

12. Morris, J. C., Neben, S., Bennett, F., Finnerty, H., Long, A., Beier, D. R., Kovacic, S., McCoy, J. M., DiBlasio-Smith, E., **LaVallie, E. R.,** Calvetti, J., Morris, G., Weich, N., Paul, S. R., Crosier, P. S., Turner, K. J., and Wood, C. R. 1996. Molecular cloning and characterization of murine interleukin-11. Exp. Hematol. 24, 1369-1376.

13. Lu, Z., DiBlasio-Smith, E. A., Grant, K. L., Warne, N. W., **LaVallie, E. R.,** Collins-Racie, L. A., Follettie, M. T., Williamson, M. J., and McCoy, J. M. 1996. "Histidine-patch" thioredoxins: Mutant forms of thioredoxin with metal chelating affinity that provide for convenient purifications of thioredoxin fusion proteins. J. Biol. Chem. 271 (9), 5059-5065.

14. Vozza, L. A., Wittwer, L., Higgins. D. R., Purcell, T. J., Bergseid, M., Collins-Racie, L. A., **LaVallie, E. R.,** and Hoeffler, J. P. 1996. Production of a recombinant bovine enterokinase catalytic subunit in the methylotrophic yeast Pichia pastoris. Bio/Technology 14, 77-81.

15. Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., Kriz, R., **LaVallie, E.,** Merberg, D., Spaulding, V., Stover, J., Williamson, M., and McCoy, J. 1997. A genetic selection for isolating cDNAs encoding secreted proteins. Gene 198, 289-296.

16. Smith, P. A., Tripp, B. C., DiBlasio-Smith, E. A., Lu, Z., **LaVallie, E. R.,** and McCoy, J. M. 1998. A plasmid expression system for quantitative in vivo biotinylation of thioredoxin fusion proteins in Escherichia coli. Nucleic Acids Res. 26 (6), 1414-1420.

17. Sun, B., Bush, S., Collins-Racie, L. A., **LaVallie, E. R.,** DiBlasio-Smith, E. A., Wolfman, N. M., McCoy, J. M., and Sive, H. 1999. Derrière: a novel TGFb family member required for posterior determination in Xenopus. Development 126(7):1467-82.

18. Mi, S., Lee, X., Li, X.-P., Veldman, G. M., Finnerty, H., Racie, L., **LaVallie, E.,** Edouard, P., Howes, S., Keith, J., and McCoy, J. M. 2000. Syncytin, a captured retroviral envelope protein involved in human placental morphogenesis. Nature 403:785-9.

19. Hick, E., Sun, B., Collins-Racie, L., **LaVallie, E.,** and Sive, H. 2001. Identification and characterization of fullback, a novel posteriorly-expressed Xenopus gene. Development, submitted.

20. Bradley, L., Sun, B., Collins-Racie, L., **LaVallie, E.,** McCoy, J., and Sive, H. 2000. Different activities of the frizzled-related proteins frzb2 and sizzled2 during Xenopus anteroposterior patterning. Development 227(1):118-32.

21. **LaVallie, E. R.,** Lu, Z., DiBlasio-Smith, E. A., Collins-Racie, L. A., and McCoy, J. M. 2000. Thioredoxin as a fusion partner for soluble recombinant protein production in Escherichia coli. Meth. Enzymol. 326:322-40.

22. Smith, D. M., Collins-Racie, L. A., Marigo, V. A., Roberts, D. J., Davis, N. M., Hartmann, C., Schweitzer, R., **LaVallie, E. R.,** Gamer, L., McCoy, J., and Tabin, C. J. 2001. Cloning and expression of a novel cysteine-rich secreted protein family member expressed in thyroid and pancreatic mesoderm within the chicken embryo. Mech. Development 102:223-226.

23. Crosier PS, Bardsley A, Horsfield JA, Krassowska AK, **LaVallie ER,** Collins-Racie LA, Postlethwait JH, Yan YL, McCoy JM, Crosier KE. 2001 In situ hybridization screen in zebrafish for the selection of genes encoding secreted proteins. Dev Dyn. 222(4):637-44.

24. Westling J, Fosang AJ, Last K, Thompson VP, Tomkinson KN, Hebert T, McDonagh T, Collins-Racie LA, **LaVallie ER,** Morris EA, Sandy JD. 2002. ADAMTS4 cleaves at the aggrecanase site (Glu373-Ala374) and secondarily at the matrix metalloproteinase site (Asn341-Phe342) in the aggrecan interglobular domain. J Biol Chem. 277(18):16059-66.

25. Horsfield J, Ramachandran A, Reuter K, **LaVallie E,** Collins-Racie L, Crosier K, Crosier P. 2002 Cadherin-17 is required to maintain pronephric duct integrity during zebrafish development. Mech Dev. 115(1-2):15-26.

26. Flannery CR, Zeng W, Corcoran C, Collins-Racie LA, Chockalingam PS, Hebert T, Mackie SA, McDonagh T, Crawford TK, Tomkinson KN, **LaVallie ER,** Morris EA. 2002. Autocatalytic cleavage of ADAMTS-4 (Aggrecanase-1) reveals multiple glycosaminoglycan-binding sites. J Biol Chem. 277(45):42775-80.

27. **LaVallie ER**, DiBlasio-Smith EA, Collins-Racie LA, Lu Z, McCoy JM. 2003. Use of thioredoxin and related proteins as multi-functional fusion tags for soluble expression in E. coli. In: Methods in Molecular Biology, vol. 205:119-140, *E. coli* Gene Expression Protocols. P. E. Vaillancourt, ed. Humana Press Inc., Totowa, NJ.

28. Lu Z, **LaVallie ER**, McCoy JM. 2003. Using bio-panning of FLITRX peptide libraries displayed on E. coli cell surface to study protein-protein interactions. In: Methods in Molecular Biology, vol. 205:267-280, *E. coli* Gene Expression Protocols. P. E. Vaillancourt, ed. Humana Press Inc., Totowa, NJ.

Issued U. S. Patents

1. Stahl,M.L., and **LaVallie, E.R.** (1989) United States Patent No. 4,801,536 Issued January 31, 1989. "Method for producing heterologous proteins."

2. McCoy, J.M., and **LaVallie, E.R.** (1993) United States Patent No. 5,270,181 Issued December 14, 1993. "Peptide and Protein Fusions to Thioredoxin and Thioredoxin-like Molecules."

3. McCoy, J.M., and **LaVallie, E.R.** (1994) United States Patent No. 5,292,646 Issued March 8, 1994. "Peptide and Protein Fusions to Thioredoxin and Thioredoxin-like Molecules."

4. McCoy, J., DiBlasio, E., Grant, K., and **LaVallie, E.R.** (1994) United States Patent No. 5,646,016. Issued July 8, 1997. "Peptide and Protein Fusions to Thioredoxin, Thioredoxin like Molecules, and Modified Thioredoxin-like Molecules."

5. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V. (1997) United States Patent No. 5,654,173. Issued August 5, 1997. "Secreted Proteins and Polynucleotides Encoding Them."

6. **LaVallie, E. R.** (1997) United States Patent No. 5,665,566. Issued September 9, 1997. "Cloning of Enterokinase and Method of Use."

7. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Evans, C., Spaulding, V. (1998) United States Patent No. 5,708,157. Issued January 13, 1998. "Secreted Proteins and Polynucleotides Encoding Them."

8. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V. (1998) United States Patent No. 5,723,315. Issued March 3, 1998. "Secreted Proteins and Polynucleotides Encoding Them."

9. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Evans, C., Spaulding, V. (1998) United States Patent No. 5,728,819. Issued March 17, 1998. "Secreted Proteins and Polynucleotides Encoding Them."

10. Vicik, S., Schauer, N., Mercer, J., **LaVallie, E.,** Briasco, C., Deetz, J., Winters, D., and Thomas, J. (1998) United States Patent #5,760,189. Issued June 2, 1998. "Novel Protein Recovery and Purification Methods."

11. Jacobs, K., McCoy, J. M., Racie, L. A., **LaVallie, E. R.,** and Merberg, D. (1996) United States Patent Application USSN 08/628,364 "Polynucleotides Encoding Secreted Proteins."

12. McCoy, J., DiBlasio-Smith, E., Grant, K., and **LaVallie, E. R.** (1997) United States Patent No. 5,646,016. Issued July 8, 1997. "Peptide and Protein Fusions to Thioredoxin, Thioredoxin-like Molecules, and Modified Thioredoxin-like Molecules."

13. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V. (1998) United States Patent No. 5,786,465. Issued July 28, 1998. "Secreted Proteins and Polynucleotides Encoding Them."

14. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Evans, C., Spaulding, V., Bowman, M. (1998) United States Patent No. 5,807,703. Issued September 15, 1998. "Secreted Proteins and Polynucleotides Encoding Them."

15. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V. (1998) United States Patent No. 5,807,709. Issued September 15, 1998. "Secreted Proteins and Polynucleotides Encoding Them."

16. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V. (1998) United States Patent No. 5,827,688. Issued October 27, 1998. "Secreted Proteins and Polynucleotides Encoding Them."

17. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Bowman, M., Spaulding, V. (1998) United States Patent No. 5,831,056. Issued November 17, 1998. "Secreted Proteins and Polynucleotides Encoding Them."

18. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V. (1998) United States Patent No. 5,837,490. Issued November 3, 1998. "Secreted Proteins and Polynucleotides Encoding Them."

19. **LaVallie, E. R.,** Racie, L. A., DeRobertis, E. M. (1998) United States Patent No. 5,846,770. Issued December 8, 1998. "DNA Molecules Encoding Chordin."

20. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D. (1999) United States Patent No. 5,945,302. Issued August 31, 1999. "Polynucleotides Encoding Secreted Proteins."

21. Jacobs, K., McCoy, J. M., Racie, L. A., **LaVallie, E. R.,** Merberg, D., Treacy, M., Evans, C. (1999) United States Patent No. 5,958,726. Issued September 28, 1999. "Secreted Proteins and Polynucleotides Encoding Them."

22. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Evans, C., Bowman, M. (1999) United States Patent No. 5,965,388. Issued October 12, 1999. "Secreted Proteins and Polynucleotides Encoding Them."

23. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V., Agostino, M. (1999) United States Patent No. 5,965,397. Issued October 12, 1999. "Secreted Proteins and Polynucleotides Encoding Them."

24. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Evans, C. (1999) United States Patent No. 5,965,693. Issued October 12, 1999. "Secreted Proteins and Polynucleotides Encoding Them."

25. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V. (1999) United States Patent No. 5,969,125. Issued October 19, 1999. "Secreted Proteins and Polynucleotides Encoding Them."

26. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D. (1999) United States Patent No. 5,972,652. Issued October 26, 1999. "Polynucleotides Encoding Secreted Proteins."

27. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V., Agostino, M. (1999) United States Patent No. 5,976,837. Issued November 2, 1999. "Secreted Proteins and Polynucleotides Encoding Them."

28. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V., Agostino, M. (1999) United States Patent No. 5,976,838. Issued November 2, 1999. "Secreted Proteins and Polynucleotides Encoding Them."

29. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V. (1999) United States Patent No. 5,981,222. Issued November 9, 1999. "Human semaphorin E, secreted proteins and polynucleotides encoding them ."

30. Jacobs, K., McCoy, J. M., **LaVallie, E. R.,** Racie, L. A., Merberg, D., Treacy, M., Spaulding, V., Evans, C. (1999) United States Patent No. 5,985,602. Issued November 16, 1999. "Secreted Proteins and Polynucleotides Encoding Them."

31. **LaVallie, E. R.,** Racie, L. A., DeRobertis, E. M. (1999) United States Patent No. 5,986,056. Issued November 16, 1999. "Chordin Compositions."

32. Racie, L. A., Jacobs, K., Lu, Z., **LaVallie, E. R.**, McCoy, J. M. (2000) United States Patent No. 6,084,071. Issued July 4, 2000. "Human L105 polypeptides and polynucleotides encoding same."

33. McCoy, J., DiBlasio-Smith, E., Grant, K., and **LaVallie, E. R.** (2000) United States Patent No. 6,143,524. Issued November 7, 2000. "Peptide and protein fusions to thioredoxin, thioredoxin-like molecules, and modified thioredoxin-like molecules."

34. Racie; L., **LaVallie, E.**, Paulsen, J., Sive; H., and Sun, B. (2000) United States Patent No. 6,165,748. Issued December 26, 2000. "Frazzled nucleotide sequences and expression products."

35. Jacobs, K., McCoy, J. M., Racie, L.A., **LaVallie, E.R.,** Merberg, D., Treacy, M., and Evans, C. (2001) United States Patent No. 6,280,739. Issued August 28, 2001. "Method of inhibiting angiogenesis using secreted proteins."

36. Jacobs, K., McCoy, J.M., **LaVallie, E.R.,** Racie, L.A., Evans, C., Merberg, D., Mi, S., and Treacy, M. (2001) United States Patent No. 6,312,921. Issued November 6, 2001. "Secreted proteins and polynucleotides encoding them."

37. Jacobs, K., McCoy, J.M., Racie, L.A., **LaVallie, E.R.,** Merberg, D., Treacy, M., Spaulding, V., and Agostino, M.J. (2002) United States Patent No. 6,451,318. Issued September 17, 2002.

Abstracts and Presentations

1. McColgan, J.M., **LaVallie, E.R.,** Murray, B.L., McCoy, J.M., Schendel, P.F., and Schauer, N.L. A novel and efficient plasmid maintenance system for long term fermentation of Escherichia coli. Presented at American Chemical Society Annual Meeting, Washington, D.C., March 1990.

2. **LaVallie, E.R.***, McCoy, J.M., DiBlasio, E.A., and Schendel, P.F. Control of E. coli aminopeptidases to produce homogeneous N-terminal sequences. *Invited speaker at American Society for Microbiology Biotechnology Conference, New York City, June 1991.

3. **LaVallie, E.R.,** DiBlasio, E.A., Kovacic, S., Grant, K.L., Schendel, P.F., and McCoy, J.M. An E. coli expression system that allows high-level soluble production of heterologous proteins. Presented at Bio/Technology Miami Winter Symposia, Miami, FL, January 1993.

4. **LaVallie, E.R.,** Rehemtulla, A., Racie, L.A., DiBlasio-Smith, E.A., Ferenz, C., Light, A., and McCoy, J.M. Recombinant bovine enterokinase: expression, purification, and comparison with the native heterodimeric enzyme. Presented at the Keystone Symposium on Structural and Molecular Biology of Protease Function and Inhibition, Santa Fe, New Mexico, March 1994.

5. DiBlasio-Smith, E.A., Grant, K.L., Wolfman, N., **LaVallie, E.R.,** Racie, L.A., and McCoy, J.M. Cloning of the gene coding for a new E. coli outer membrane protease. Presented at the Keystone Symposium on Structural and Molecular Biology of Protease Function and Inhibition, Santa Fe, New Mexico, March 1994.

6. Lu, Z., van Cleave, V., Murray, K.S., **LaVallie, E.R.**, Stahl, M.L., and McCoy, J.M. Display of Thioredoxin Random Peptide Libraries on the Escherichia coli Cell Surface: A System for Exploring Protein-Protein Interactions
and its Applications in Epitope Mapping. Presented at the Protein Engineering Methodologies for Drug Design Symposium, San Diego, CA, June 27-28, 1994.

7. **LaVallie, E.R.*,** DiBlasio-Smith, E.A., Racie, L.A., Kovacic, C., Grant, K.L., Schendel, P.F., and McCoy, J.M. Soluble production of eukaryotic proteins in Escherichia coli using thioredoxin fusions and recombinant enterokinase. *Invited speaker at Society for Industrial Microbiology Annual Meeting, Boston, MA, August 1994.

8. Lu, Z., DiBlasio-Smith, L., Grant, K., Warne, N., **LaVallie, E.,** Racie, L., Follettie, M., Williamson, M., and McCoy, J. "Histidine-patch" thioredoxins: mutant forms of thioredoxin with metal chelating affinity which provide for convenient purifications of thioredoxin fusion proteins. Prot. Sci. 4 (Suppl. 2), July 1995, p. 83. Presented at the Protein Society Ninth Symposium, Boston, MA, July 8, 1995.

9. Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., Kriz, R., **LaVallie, E.,** Merberg, D., Spaulding, V., Stover, J., Williamson, M., and McCoy, J. A genetic selection for isolating cDNA's encoding secreted proteins. Presented at the Genome Sequencing Conference, Hilton Head, SC, October, 1996.

10. Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., Kriz, R., **LaVallie, E.,** Merberg, D., Spaulding, V., Stover, J., Williamson, M., and McCoy, J. A genetic selection for isolating cDNA's encoding secreted proteins. Presented at the Functional Genomics Conference, San Diego, CA, December 1996.

11. Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., Kriz, R., **LaVallie, E.,** Merberg, D., Spaulding, V., Stover, J., Williamson, M., and McCoy, J. A genetic selection for isolating cDNA's encoding secreted proteins. Presented at the Keystone Symposium entitled "Discovery and Development of Novel Therapeutic Agents for the 21st Century", Tamarron, CO, March 1997.

12. Bradley, L., Bush, S., Sive, H., Racie, L., **LaVallie, E.,** and McCoy, J. Frzbs and Patterning in *Xenopus laevis*. Presented at the Harvard University Wnt Meeting 1998, January 1998.

13. Georgiadis K, Crawford T, Tomkinson K, Shakey Q, Morris E, Collins-Racie L, **LaVallie E.** ADAMTS-5 is autocatalytic at a E753-G754 site in the spacer domain.

Presented at the Orthopaedic Research Society Annual Meeting, San Francisco, CA, February 2002.

14. Arai, M., Anderson, D., Freeman, B., Collins-Racie, L., Corcoran, C., DiBlasio-Smith, E., Morris, E., and **LaVallie, E.R.** Cloning and characterization of bovine Aggrecanase-1. Presented at the Osteoarthritis Research Society International Annual Meeting, Sydney, Australia, September 2002.

References available upon request.

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 6770–6775, June 1997
Developmental Biology

Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action

PAUL W. FINCH*, XI HE†‡, MICHAEL J. KELLEY§, AYKUT ÜREN¶, R. PAUL SCHAUDIES¶‖, NICHOLAS C. POPESCU**, STUART RUDIKOFF††, STUART A. AARONSON*, HAROLD E. VARMUS†, AND JEFFREY S. RUBIN¶‡‡

*Derald H. Ruttenberg Cancer Center, Mount Sinai Medical Center, New York, NY 10029-6574; and †Varmus Laboratory, §Medicine Branch, and Laboratories of ¶Cellular and Molecular Biology, **Experimental Carcinogenesis, and ††Genetics, National Cancer Institute, Bethesda, MD 20892

Contributed by Harold E. Varmus, April 15, 1997

ABSTRACT **Frizzled polypeptides are integral membrane proteins that recently were shown to function as receptors for Wnt signaling molecules. Here, we report the identification of a novel, secreted 36-kDa protein that contains a region homologous to a putative Wnt-binding domain of Frizzleds. This protein, called Frizzled-related protein (FRP), was first identified as a heparin-binding polypeptide that copurified with hepatocyte growth factor/scatter factor in conditioned medium from a human embryonic lung fibroblast line. Degenerate oligonucleotides, based on the NH_2-terminal sequence of the purified protein, were used to isolate corresponding cDNA clones. These encoded a 313-amino acid polypeptide, containing a cysteine-rich domain of ≈110 residues that was 30–40% identical to the putative ligand-binding domain of Frizzled proteins. A 4.4-kb transcript of the *FRP* gene is present in many organs, both in the adult and during embryogenesis, and homologs of the gene are detectable in DNA from several vertebrate species. In biosynthetic studies, FRP was secreted but, like Wnts, tended to remain associated with cells. When coexpressed with several Wnt family members in early *Xenopus* embryos, FRP antagonized Wnt-dependent duplication of the embryonic dorsal axis. These results indicate that FRP may function as an inhibitor of Wnt action during development and in the adult.**

Extracellular signaling molecules have essential roles as inducers of cellular proliferation, migration, differentiation, and tissue morphogenesis during normal development. They also participate in many of the aberrant growth regulatory pathways associated with neoplasia. Among the molecules involved in these activities are the Wnt glycoproteins. In vertebrates, this family consists of more than a dozen structurally related molecules, containing 350–380 amino acid residues of which >100 are conserved, including 23–24 cysteine residues (1, 2). *Wnt-1*, the first Wnt-encoding gene to be isolated, was identified as an oncogene expressed as a result of insertional activation by the mouse mammary tumor virus (3, 4). Subsequently, transgenic expression of *Wnt-1* confirmed that constitutive expression of this gene caused mammary hyperplasia and adenocarcinoma (5). Targeted disruption of the *Wnt-1* gene revealed an essential role in development, as mouse embryos had severe defects in their midbrain and cerebellum (6–8). *Wingless* (*Wg*), the *Drosophila* homolog of *Wnt-1*, was independently identified as a segment polarity gene (9). Gene targeting of other *Wnt* genes demonstrated additional important roles for these molecules in kidney tubulogenesis and limb bud development (10, 11).

Several aspects of Wnt signaling have been illuminated by studies in flies, worms, frogs, and mice (12, 13), but until recently little was known about key events that occur at the external cell surface. Identification of Wnt receptors was hampered by the relative insolubility of the Wnt proteins, which tend to remain tightly bound to cells or extracellular matrix (14, 15). However, several observations now indicate that members of the Frizzled (FZ) family of molecules (16) can function as receptors for Wnt proteins or as components of a Wnt receptor complex (17–19). The prototype of this family, *Drosophila frizzled* (*Dfz*), was first identified as a tissue polarity gene that governs orientation of epidermal bristles (20). Cells programmed to express a second *Drosophila Fz* gene, *Fz2*, bind Wg and transduce a Wg signal to downstream components of the signaling pathway (17).

Each *Fz* gene encodes an integral membrane protein with a large extracellular portion, seven putative transmembrane domains, and a cytoplasmic tail (16, 21). Near the NH_2 terminus of the extracellular portion is a cysteine-rich domain (CRD) that is well conserved among other members of the FZ family (16). The CRD, comprised of ≈110-amino acid residues, including 10 invariant cysteines, is the putative binding site for Wnt ligands (17).

Given the potential complexity of interactions between the multiple members of Wnt and FZ families (1, 16–19), additional mechanisms might exist to modulate Wnt signaling during specific periods of development or in certain tissues. Here we report evidence for such a mechanism, namely the identification of a novel secreted gene product that is closely related to the FZ CRD and antagonizes Wnt action. We propose that this FRP is a prototype for molecules that function as endogenous regulators of Wnt activity.

MATERIALS AND METHODS

Purification and Physical Characterization. Conditioned-medium collection, ultrafiltration, heparin-Sepharose affinity chromatography, and SDS/PAGE were performed as described (22). Hepatocyte growth factor/scatter factor (HGF/SF)-containing fractions were identified by immunoblotting (23). Occasionally heparin-Sepharose fractions were processed by reverse-phase C_4 HPLC (22) to enhance purity of FRP. Gels were fixed and silver-stained using the reagents and protocol from Bio-Rad.

Microsequencing. Approximately 30 μg of protein was loaded onto an Applied Biosystems gas-phase protein sequenator. Forty rounds of Edman degradation were carried out, and phenylthiohydantoin amino acid derivatives were identified with an automated on-line HPLC column (model 120A, Applied Biosystems).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FRP, Frizzled-related protein; FZ, Frizzled; CRD, cysteine-rich domain of Frizzled proteins; HGF/SF, hepatocyte growth factor/scatter factor.

Data deposition: The sequence in this paper has been deposited in the GenBank database (accession no. AF001900).

‡Present address: Division of Neuroscience, Children's Hospital/Harvard Medical School, Boston, MA 02115.
‖Present address: Defense Intelligence Agency, Arlington VA, 22201.
‡‡To whom reprint requests should be addressed at: National Cancer Institute, Laboratory of Cellular and Molecular Biology, Building 37, Room 1E24, 37 Convent Drive, Medical Science Center 4255, Bethesda, MD 20892-4255. e-mail: rubinj@dc37a.nci.nih.gov.

Molecular Cloning and Analysis. Four pools of 26-base degenerate oligonucleotides were synthesized on the basis of either of two segments of amino acid sequence determined by microsequencing of purified FRP. Two pools corresponding to the sequence NVGYKKMVL contained all possible codon combinations except for the substitution of inosine residues in the third positions of the codons for the first Val and Gly; one subset terminated with bases CT and the other with TT. Two additional pools, corresponding to the sequence FYTKPPQXV, contained all possible codon combinations except for the substitution of inosine residues in the third positions of the codons for both Pro residues; one subset contained four codon options for serine in the X position, while the other had the remaining two. Oligonucleotide pools were labeled and used to screen an oligo(dT)-primed M426 cDNA library as described (24).

Selected cDNA inserts were analyzed by restriction endonuclease digestion. The nucleotide sequence of the FRP cDNAs was determined by the dideoxy chain-termination method. To search for homology between FRP and any known protein, we analyzed the GenBank, Protein Data Base, Swiss-Prot, and Protein Identification Resource protein sequence databases. Alignments were generated with the program PILEUP 8 from the Wisconsin Package (Genetics Computer Group, Madison, WI).

Northern and Southern Blotting Analyses. RNA from cell lines was isolated, transferred to nitrocellulose filters, and hybridized with labeled probes as described (24). Northern blots containing ≈2 μg of poly(A)$^+$ RNA isolated from a variety of different organs were purchased from Clontech. Labeled probes were hybridized in Express Hyb hybridization solution (Clontech) according to the manufacturer's protocol. The FRP *NotI–SmaI* cDNA fragment and human β-actin cDNA probe provided by Clontech were ^{32}P-labeled with random hexamers and used at a concentration of 1–2 × 10^6 cpm/ml (specific activity >8 × 10^8 cpm/μg DNA).

Southern blot analysis was performed as described (25), except for variation in formamide concentration during hybridization, as noted in the text. FRP cDNA probes were ^{32}P-labeled with the nick-translation kit from Amersham.

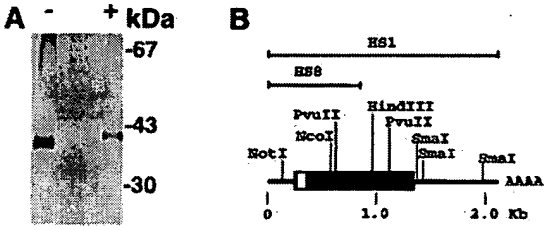
Chromosomal Localization. A 4.1-kb *FRP* genomic fragment obtained from a human fibroblast genomic DNA library (Stratagene) was labeled with biotin or digoxigenin and used as a probe for *in situ* hybridization to locate the *FRP* gene in chromosomal preparations of methotrexate-synchronized normal peripheral human lymphocyte cultures. The conditions for hybridization, detection of fluorescent signal, digital-image acquisition, processing, and analysis were as described (26). The identity of the chromosomes with specific signal was confirmed by rehybridization using a chromosome 8-specific probe, and the signal was localized on G-banded chromosomes.

Biosynthetic Studies. M426 cells grown in T-25 flasks were incubated for 30 min in methionine-free DMEM in the presence or absence of 50 μg/ml heparin (bovine lung, Sigma; when present, heparin was included in all subsequent media), which was subsequently replaced with medium containing [^{35}S]methionine (1 mCi/5 ml per dish; 1 Ci = 37 GBq). After 30 min, the radioactive medium was removed, and monolayers washed with medium containing unlabeled methionine, then incubated for varying intervals in fresh nonradioactive medium. At the specified times, the conditioned media and cell lysates were collected and processed as described (27). Immunoprecipitations were performed with a rabbit polyclonal antiserum (100 μg/ml) raised against a synthetic peptide corresponding to FRP amino acid residues 41–54, in the presence or absence of competing peptide (50 μg/ml). Immune complexes adsorbed to GammaBind (Pharmacia) were pelleted by centrifugation and washed; labeled proteins were resolved by SDS/PAGE and detected by autoradiography.

Microinjection of mRNAs into *Xenopus* Embryos. Wnt-1, wg, Xwnt-3a, and Xwnt-8 plasmids were used as described (28–31). The FRP *NaeI–SalI* cDNA fragment, which includes the full coding sequence, was subcloned into the *StuI* and *XhoI* sites of pCS2+ (32). All mRNAs for injection were synthesized as capped transcripts *in vitro* with SP6 RNA polymerase (Ambion Megascript Kit). Embryo preparation and staging were performed as described (33). Transcripts were injected into the two blastomeres near the equatorial midline region at the 4-cell stage.

RESULTS

Purification and Molecular Cloning of a Novel Heparin-Binding Protein. During the isolation of HGF/SF from human embryonic lung fibroblast culture fluid, we identified a 36-kDa polypeptide that copurified with HGF/SF following a variety of chromatography procedures (27). Because the comigration of this protein and HGF/SF suggested that it might regulate growth factor activity, a preparative scheme was devised to obtain sufficient quantities for study. This was accomplished by conservative pooling of fractions eluting from heparin-Sepharose resin with 1.0 M NaCl, once it became evident that a portion of the 36-kDa protein emerged after the HGF/SF-containing fractions. Protein obtained in this manner was sufficiently pure and abundant for structural and limited functional analysis (Fig. 1*A*).



C

```
                10                  20                  30                  40
MGIGRSEGGRRGALGVLLALGAALLAVGSASEYDYVSFQS

                50                  60                  70                  80
DIGPYQSGRFYTKPPQCVDIPADLRLCHNVGYKKMVLPNL

                90                 100                 110                 120
LEHETMAEVKQQASSWVPLLNKNCHAGTQVFLCSLFAPVC

               130                 140                 150                 160
LDRPIYPCRWLCEAVRDSCEPVMQFFGFYWPEMLKCDKFP

               170                 180                 190                 200
EGDVCIAMTPPNATEASKPQGTTVCPPCDNELKSEAIIEH

               210                 220                 230                 240
LCASEPALRMKIKEVKKENGDKKIVPKKKKPLKLGPIKKK

               250                 260                 270                 280
DLKKLVLYLKNGADCPCHQLDNLSHHFLIMGRKVKSQYLL

               290                 300                 310
TAIHKWDKKNKEFKNFMKKMKNHECPTPQSVFK
```

FIG. 1. (*A*) SDS/PAGE analysis of heparin-Sepharose purified FRP. Approximately 200 ng of protein was resolved in a 4–20% polyacrylamide minigel (Novex) under reducing (+) or nonreducing (−) conditions, and subsequently stained with silver. The position of molecular mass markers is indicated at the right. (*B*) Representation of human FRP cDNA clones. Overlapping clones HS1 and HS8 are shown above a diagram of the complete coding sequence and the adjacent 5′ and 3′ untranslated regions. The coding region is boxed; the open portion corresponds to the signal sequence. Untranslated regions are represented by a line. Selected restriction sites are indicated. (*C*) Predicted FRP amino acid sequence (standard single-letter code). The peptide sequence obtained from the purified protein is underlined. Double-underlined sequences were used to generate oligonucleotide probes for screening of the M426 cDNA library. The putative signal sequence is italicized. The large shaded region is the cysteine-rich domain homologous to CRDs in members of the FZ family. The small shaded region is a lysine-rich segment that fulfills the criteria for a consensus hyaluronic acid-binding sequence. The dashed underlining denotes two potential asparagine-linked glycosylation sites.

Microsequencing of the purified 36-kDa protein yielded two NH_2-terminal sequences, one beginning three residues downstream from the other. Positive identifications were made in 37 of the first 40 cycles of Edman degradation, as follows: FQSDIGPYQSGRFYTKPPQXVDIPADLRLXXNVGYKKMVL (X denotes inability to make an amino acid assignment). Degenerate oligonucleotides corresponding either to sequence FYTKPPQXV or NVGYKKMVL were used to probe a M426 cDNA library. An initial screening of 10^6 plaques yielded ≈350 clones recognized by probes derived from both peptide segments. Restriction digestion of several plaque-purified phage DNAs revealed two classes of inserts. Mapping (Fig. 1*B*) and sequence analysis (Fig. 1*C*) of a representative from each class, designated HS1 and HS8, demonstrated that they were overlapping cDNAs. HS1 was ≈2 kb in length and contained a 942-bp ORF; HS8 encoded a portion of the 942-bp ORF as well as ≥0.3 kb of cDNA extending upstream of the ATG start codon. The putative start codon, located at position 303 in the HS1 sequence, was flanked by sequence that closely matched the proposed GCC(G/A)CCATGG consensus sequence for optimal initiation by eukaryotic ribosomes (34). An upstream in-frame stop codon was not present.

As expected for a secreted protein, a hydrophobic 27-amino acid segment at the NH_2-terminus likely functions as a signal peptide. The experimentally determined protein sequence begins 11 residues downstream from the presumptive signal sequence, suggesting additional processing or incidental proteolysis. There was complete agreement between the predicted and observed amino acid sequences; the three undefined residues in the latter corresponded to Cys-57, Cys-67, and His-68, residues that typically are undetectable or have low yields following Edman degradation. Two overlapping sequences in the COOH-terminal region fulfill the criteria for a consensus binding site to hyaluronic acid (35) (Fig. 1*C*). Two potential asparagine-linked glycosylation sites are also present. A consensus polyadenylylation signal was not identified in the cDNA sequence, raising the possibility that the cDNA clones from this oligo(dT) primed library resulted from internal priming at an adenine-rich region.

Relationship to FZ. Search of several protein databases revealed significant homology of a portion of the predicted amino acid sequence to a specific region conserved among members of the FZ family (Fig. 2). The observed homology is confined to the extracellular CRD of FZ, a region consisting of ≈110 amino acid residues that includes 10 cysteines and a small number of other invariant residues. This domain has special importance because it is a putative binding site for Wnt ligands (17). The FRP CRD is 30–42% identical to the CRD of the other FZ proteins.

In addition to the plasma membrane-anchored FZ proteins and FRP, three other molecules have been described that also possess a FZ CRD motif. An alternatively spliced isoform of mouse collagen XVIII was the first such protein to be reported (38). The two other molecules, mouse SDF5 (40) and human FRZB (39), resemble FRP in that each consists of ≈300 amino acid residues, including a signal peptide, CRD near its NH_2-terminus and a hydrophilic COOH-terminal moiety. FRP and SDF5 have 58% identities in their CRDs, while FRP and FRZB are only 32% identical in this region. Elsewhere, these molecules are only 15–20% identical. Thus, FRP, SDF5, and FRZB may constitute a subfamily of small, FZ-related proteins that lack the seven transmembrane motif responsible for anchoring FZ proteins to the plasma membrane and are presumably secreted.

FRP Gene is Expressed in Multiple Organs and Cell Types. Using the 1081-bp *Not*I–*Sma*I fragment of HS1 (Fig. 1*B*) as probe, a single 4.4-kb transcript was detected in poly(A)$^+$ RNA from several human organs (Fig. 3). In adult tissues, the highest level of expression was observed in heart, followed by kidney, ovary, prostate, testis, small intestine, and colon. Lower levels were seen in placenta, spleen and brain, while transcript was barely detectable in skeletal muscle and pancreas. No hybridization signal was evident in mRNA from lung, liver, thymus, or peripheral blood leukocytes. In poly(A)$^+$ RNA from a small sample of human fetal organs, the 4.4-kb transcript was highly

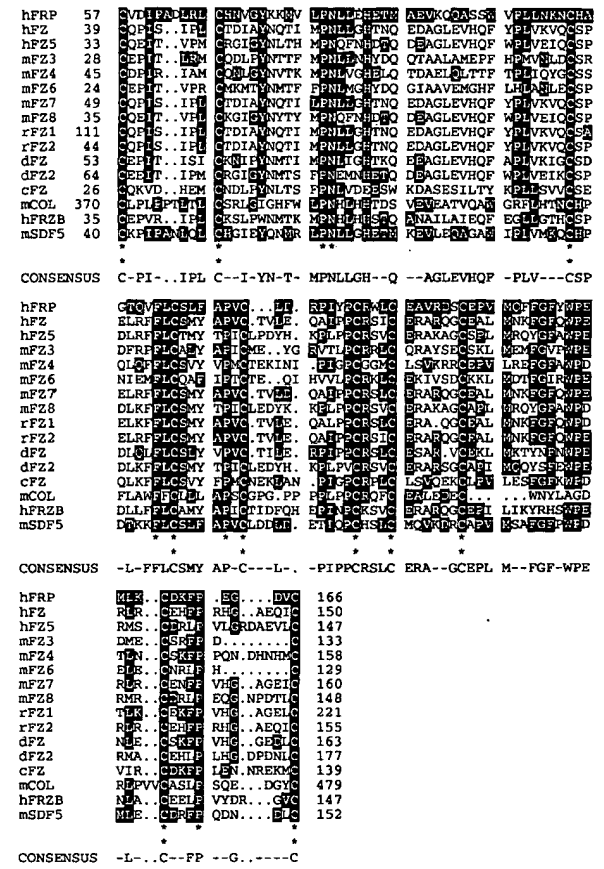


FIG. 2. Comparison of the CRDs of FRP and other members of the FZ family. Solid black shading highlights identities present in human FRP and any other FZ family member. The consensus sequence indicates residues present in at least 8 of the 16 FZ or FZ-related proteins. **, The ten invariant cysteine residues; single asterisks indicate other invariant residues. hFRP, human FZ-related protein; hFZ (36); hFZ5 (16); mFZ3-mFZ8 (16); rFZ1 and rFZ2 (37); dFZ (21); dFZ2 (17); cFZ (16); mCOL, mouse collagen XVIII (38); hFRZB (39); and mSDF5 (40).

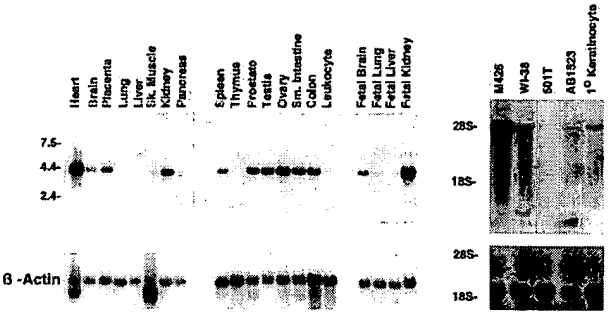


FIG. 3. FRP mRNA expression in normal human adult and embryonic tissues, and in cultured cells. Blots containing ≈2 μg of poly(A)+ RNA from each of the indicated tissues or 10 μg of total RNA from different human cell lines were probed with radiolabeled FRP and β-actin cDNA fragments, as described in the Methods. The position of DNA size markers, expressed in kb, is indicated at the left of the tissue blots; the position of 28S and 18S ribosomal RNA is shown at the left of the cell line blot.

represented in kidney, at moderate levels in brain, barely detectable in lung, and undetectable in liver.

Northern analysis of total RNA from various human cell lines demonstrated the 4.4-kb transcript and, occasionally, additional faint bands not further analyzed (Fig. 3 *Right*). While the transcript was detected in RNA from embryonic lung (M426 and WI-38) and neonatal foreskin (AB1523) fibroblasts, it was not observed in a sample of adult dermal fibroblasts (501T). In addition to fibroblasts, the transcript was seen in RNA from primary keratinocytes, indicating that expression was not limited to cells of mesenchymal origin. Considering that the cumulative size of the overlapping FRP cDNAs was only 2.1 kb, detection of a 4.4-kb transcript reinforced the suggestion that the cDNAs were generated by internal priming at adenine-rich regions.

Chromosomal Localization and Detection of the FRP Gene in Different Species. Using a fluorescent-labeled 4.1-kb genomic fragment containing a portion of the FRP coding sequence, *in situ* hybridization revealed a single locus at chromosome 8p11.1-12 (Fig. 4). This site may be near the putative locus of the *hFZ3* gene, based on homology with the location of *mFz3* in the mouse genome (16). Radiation hybrid analysis yielded results consistent with the fluorescent *in situ* hybridization analysis (unpublished observations).

To determine whether the *FRP* gene was present in other species, genomic DNAs from various sources were fully digested with *Eco*RI and hybridized with an *Nco*I–*Sma*I cDNA fragment (Fig. 1*B*) under varying conditions of stringency (Fig. 5). Multiple bands were observed under highly stringent conditions (50% formamide) in DNA from human, rhesus monkey, mouse, and chicken. With moderate stringency (35% formamide), no additional fragments were seen in the DNA from these species but fragments were detected in *Xenopus* DNA. No hybridization signal was observed with DNA from *Drosophila* or yeast (*Saccharomyces cerevisiae*) in these experiments. At low stringency (20% formamide), the background was too high to detect specific signals (data not shown). These results strongly suggested that the *frp* gene is highly conserved among vertebrates. Although these experiments did not detect an *FRP* homolog in the invertebrates, the existence of such homologs was not rigorously excluded, due to the limitations of the method.

Southern blotting performed either with the *Not*I–*Nco*I cDNA fragment (Fig. 1*B*) or with synthetic oligonucleotide probes corresponding to different portions of the FRP coding sequence, hybridized to subsets of genomic fragments detected with the *Nco*I–*Sma*I probe (data not shown). This finding and the lack of additional bands detected only under relaxed conditions (Fig. 5) suggested that highly related *FRP*-like sequences are not present

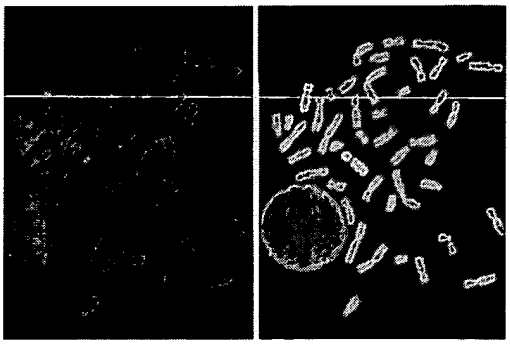


FIG. 4. Chromosomal localization of the *FRP* gene by fluorescent *in situ* hybridization. To localize the *FRP* gene, 100 sets of metaphase chromosomes were analyzed. In 80 metaphases, a double fluorescent signal was observed with the *FRP* genomic probe in 8p11.1-12 on both chromosome homologs (*Left*). The identity of the chromosomes was confirmed by hybridization with a probe specific for chromosome 8 (*Right*).

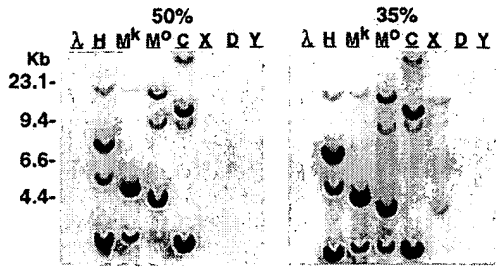


FIG. 5. Southern blot analysis of *FRP* genomic sequences in different species. After fractionation by agarose gel electrophoresis and transfer to filters, *Eco*RI-digested genomic DNAs were hybridized in the presence of either 50% or 35% formamide. Specimens were from the following species: λ, lamda phage; H, human; M^k, rhesus monkey; M^o, mouse; C, chicken; X, *Xenopus laevis*; D, *Drosophila melanogaster*; Y, yeast (*S. cerevisiae*).

in the human genome. Thus, the multiple genomic fragments hybridizing to the FRP cDNA in Southern blots are likely to reflect the presence of several exons in the *hFRP* gene.

Biosynthetic Studies Show that FRP is Secreted, but Primarily Cell-Associated in the Absence of Exogenous Heparin. To study the synthesis and processing of FRP protein, a pulse-chase experiment was performed with [^{35}S]methionine labeled M426 cells either in the absence or presence of added heparin. As shown in Fig. 6, a 36-kDa protein band was specifically immunoprecipitated with antiserum raised against a synthetic peptide corresponding to a portion of the FRP NH_2-terminal sequence. In the absence of soluble heparin, after either 1 h (lanes 1 and 5) or 4 h (lanes 9 and 13) FRP was much more abundant in the cell lysate than in the conditioned medium. However, after 20 h, the amount of FRP protein in the medium (lane 21) was comparable to that which remained cell-associated (lane 17). At this last time point, the combined band intensity in the two compartments had decreased relative to that observed earlier, suggesting significant protein turnover during the experiment. Moreover, after 20 h the FRP-specific signal appeared as a doublet, providing additional evidence of proteolysis. In the presence of soluble heparin (50 μg/ml), most of the FRP was detected in the medium at all three time points (compare lanes 3 and 7, 11 and 15, 19 and 23). Heparin also appeared to stabilize FRP, as the band intensity was stronger when heparin was present, and there was no evidence of partial proteolysis. Interestingly, others have shown that heparin can release Wnt-1 from the cell surface in a similar manner (14, 15, 41). Taken together, our results demonstrate that FRP is secreted, although it tends to remain cell-associated and relatively susceptible to degradation unless released into the medium by soluble heparin.

FRP Antagonizes Wnt Action in *Xenopus* Embryo Assay. Because FRP possesses a potential binding site for Wnt molecules and appears to partition among cellular compartments like Wnt-1, it seemed possible that FRP might modulate the signaling activity of Wnt proteins. We envisioned two alternatives: FRP might antagonize Wnt function by binding the protein and blocking access to its cell surface signaling receptor, or FRP might enhance Wnt activity by facilitating the presentation of ligand to the FZ receptors, analogous to the action of soluble interleukin 6 receptors (42).

To test these possibilities, we examined the effect of FRP on Wnt-dependent dorsal axis duplication during *Xenopus* embryogenesis. Previous studies have demonstrated that microinjection of mRNA encoding certain Wnt molecules, such as mouse Wnt-1, Wg, XWnt-8, or XWnt-3a, into early *Xenopus* embryos can induce the formation of an ectopic Spemann organizer and, subsequently, duplication of the dorsal axis (28–31, 43, 44). As illustrated in Fig. 7, injection of suboptimal doses of Wnt-1, Wg, or XWnt-8 mRNA into embryos induced partial or complete

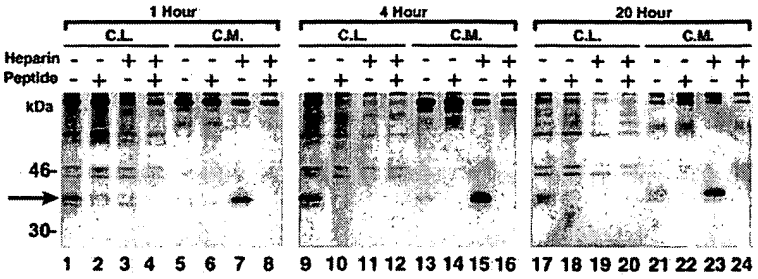


FIG. 6. Biosynthesis of FRP in M426 cells. A pulse-chase experiment was performed with metabolically labeled cells incubated either in the absence or presence of heparin. Proteins were immunoprecipitated from cell lysates (C.L.) or conditioned medium (C.M.) with FRP peptide antiserum in the absence or presence of competing peptide, and resolved in a SDS/10% polyacrylamide gel. Cells and media were harvested 1, 4, or 20 h after a 30 min labeling period. Lanes 1–24 are labeled at the bottom. The protein band corresponding to FRP is indicated by an arrow. The position of molecular mass markers is shown at the left.

duplication in at least 75% of the animals. Suboptimal doses were used to enable us to detect enhancement of the axis duplication phenotype, if the role of FRP was to facilitate Wnt signaling. However, when similar quantities of FRP and Wnt RNA were coinjected, the incidence and extent of axial duplication were significantly reduced (Fig. 7). The effect was dose-dependent, as the number of animals with an abnormal phenotype was even lower when the relative amount of FRP RNA was increased 5- to 10-fold. Injection of FRP RNA alone at a higher dose (100 pg) into the dorsal side of the embryo did not affect the endogenous dorsal axis formation (data not shown).

Surprisingly, FRP was much less effective in antagonizing XWnt-3a, suggesting a degree of specificity regarding interactions with different members of the Wnt family. The Wnt signaling pathway is thought to proceed through suppression of the activity of glycogen synthase kinase-3, a cytoplasmic serine-threonine kinase (13). Axis duplication induced by a dominant-negative, kinase-inactive mutant of glycogen synthase kinase-3β (33, 45, 46) was not affected by FRP (data not shown), consistent with the assumption that FRP directly interferes with Wnt signaling at the cell surface, not by indirectly interfering with a late step in the Wnt signaling pathway.

DISCUSSION

In the present report, we describe a novel human gene product that resembles FZ proteins in that it possesses a conserved FZ CRD, a putative binding domain for Wnt ligands. In contrast to the original members of the FZ family, FRP lacks any transmembrane region or cytoplasmic domain required to transduce Wnt signaling inside the cell. Because it is preferentially distributed to the cell surface or matrix, it is well-positioned to interact with Wnt proteins. Theoretically, binding of FRP to Wnts might stabilize the latter, and consequently enhance Wnt signaling. However, our findings indicate that in *Xenopus* embryos FRP inhibits Wnt-dependent axial duplication when various Wnts and FRP are coexpressed. These results suggest that FRP behaves like a dominant-negative receptor in this model system, similar to the effect of the secreted NH_2-terminal ectodomain of human FZ5 on axis duplication by XWnt-5A and hFZ5 (19). Further elucidation of the interaction between FRP and Wnt proteins will require study of the proteins in isolated form.

The existence of other molecules besides FRP that have a FZ CRD but lack the seven transmembrane motif and cytoplasmic tail suggests that there is a subfamily of proteins that function as regulators of Wnt activity. Little is known about the activity of SDF5, which was cloned using the signal sequence trap method (40). FRZB is a heparin-binding molecule thought to be involved in skeletal morphogenesis (39). Recently Rattner *et al.* (47) cloned cDNAs encoding the murine homologs of SDF5, FRZB, and FRP. They showed that, when artificially linked to the plasma membrane via a glycolipid anchor, SDF5 and FRZB conferred cellular binding to Wg. Thus, it now appears likely that all three molecules can interact with Wnt proteins and modulate their activity. Future studies should define the activities and unique functions of these different FZ-related proteins, especially with regard to Wnt regulation.

Recently Zecca *et al.* (48) demonstrated that Wg functions as a gradient morphogen. Just as the formation of stable Wnt gradients may rely on their ability to bind proteoglycan (41), the heparin-binding property of FRP implies that it might also function in a graded manner. In fact, gradients of interacting proteins might be a general mechanism of Wnt regulation. A detailed analysis of the expression patterns of FRP and interacting Wnt molecules would help to address the viability of this model. If FRPs and Wnts are expressed in proximity to each other, then targeted disruption of FRP expression might result in uncontrolled Wnt activity. This could be manifested by developmental abnormalities in tissue morphogenesis or perhaps by neoplasia.

The large size of the FZ and Wnt families and the potential for mutual binding between several members of the two groups make it difficult to predict which receptor/ligand interactions are likely to be affected by FRP. For example, Wg protein binds to cells expressing a variety of FZ proteins (17), yet Wnt-5A induces axis duplication only in *Xenopus* embryos expressing FZ5 (19). Furthermore, a comparison of patterns of FRP expression with published reports concerning FZs and

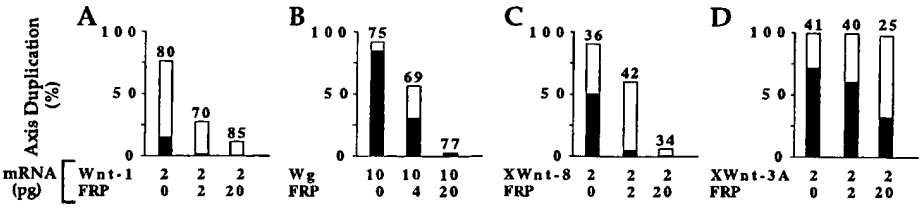


FIG. 7. Dorsal axis duplication in *Xenopus* embryos in response to varying combinations of Wnt and FRP transcripts. Each bar represents the percentage of axis duplication; the solid portion within each bar represents the percentage of extensive duplication, which is defined by the presence of the cement gland and at least one eye in the duplicated axis. The total number of embryos injected in two to four independent experiments is indicated by the number above each bar. The amount of mRNA injected per embryo is shown below the bars.

Wnts indicates that many of the *Fz* and *Wnt* genes are expressed in tissues where FRP transcripts also are seen. For instance, the heart, kidney, intestines, and reproductive organs have high levels of FRP mRNA. These organs also are among the highest in expression of FZ1, FZ2, and FZ4 transcripts (16, 37). Wnt-2, Wnt-4, Wnt-5A, and Wnt-5B have been detected at these sites as well (1). These data provide indirect evidence that FRP might have an impact on several Wnt/FZ interactions. However, knowledge about the affinity of FRP for different Wnt family members will be required to fully assess its effect on Wnt signaling. As shown in Fig. 7, FRP may inhibit only a subset of Wnt proteins.

Although our discovery of FRP was dependent on an apparent association of this molecule with HGF/SF, subsequent experiments have failed to confirm significant interaction of the two molecules. While these negative results are not definitive, they suggest that the copurification of these proteins from the same conditioned medium may have been fortuitous. Despite this apparent coincidence, the FRP and HGF/SF signaling pathways may intersect in a mutually reinforcing fashion. HGF/SF stimulates the tyrosine phosphorylation of β-catenin, which may reduce the binding of β-catenin to cadherins (13, 49). This might contribute to the disruption of cell-cell adhesion that characterizes the cell scattering activity of HGF/SF, and may antagonize aspects of Wnt-dependent cell-cell contact. In a mammary gland model, concomitant with a stimulation of branching morphogenesis, HGF/SF inhibited the expression of Wnt-5A (50). Thus, it is possible that the actions of FRP and HGF/SF complement each other in some respects, even though the proteins do not appear to have a direct interaction.

Note. While this manuscript was being reviewed, two groups reported that FRZB was a secreted antagonist of Wnt signaling expressed in the Spemann organizer (51, 52).

We thank Alex Cech, David Hirshfield, and Jared Rosenberg for technical assistance and/or help in preparing figures; and Jeremy Nathans for providing us with a copy of his manuscript (47) prior to publication. We also are grateful to Igor Dawid and Jean-Pierre Saint-Jeannet for their support and helpful discussions. P.W.F. is supported by Grant DK 47102 from the National Institutes of Health; X.H. was supported by the U.S. Army Breast Cancer Research Program.

1. Nusse, R. & Varmus, H. E. (1992) *Cell* **69,** 1073–1087.
2. Parr, B. A. & McMahon, A. P. (1994) *Curr. Opin. Genet. Dev.* **4,** 523–528.
3. Nusse, R. & Varmus, H. E. (1982) *Cell* **31,** 99–109.
4. Nusse, R., van Ooyen, A., Cox, D., Fung, Y. K. & Varmus, H. (1984) *Nature (London)* **307,** 131–136.
5. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T. & Varmus, H. E. (1988) *Cell* **55,** 619–625.
6. McMahon, A. P. & Bradley, A. (1990) *Cell* **62,** 1073–1085.
7. Thomas, K. R. & Capecchi, M. R. (1990) *Nature (London)* **346,** 847–850.
8. Thomas, K. R., Musci, T. S., Neumann, P. E. & Capecchi, M. R. (1991) *Cell* **67,** 969–976.
9. Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. & Nusse, R. (1987) *Cell* **50,** 649–657.
10. Stark, K., Vainio, S., Vassileva, G. & McMahon, A. P. (1994) *Nature (London)* **372,** 679–683.
11. Parr, B. A. & McMahon, A. P. (1995) *Nature (London)* **374,** 350–353.
12. Perrimon, N. (1996) *Cell* **86,** 513–516.
13. Miller, J. R. & Moon, R. T. (1996) *Genes Dev.* **10,** 2527–2539.
14. Papkoff, J. & Schryver, B. (1990) *Mol. Cell. Biol.* **10,** 2723–2730.
15. Bradley, R. S. & Brown, A. M. (1990) *EMBO J.* **9,** 1569–1575.
16. Wang, Y., Macke, J. P., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. & Nathans, J. (1996) *J. Biol. Chem.* **271,** 4468–4476.
17. Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. & Nusse, R. (1996) *Nature (London)* **382,** 225–230.
18. Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. J. & Moon, R. T. (1996) *Curr. Biol.* **6,** 1302–1306.
19. He, X., Saint-Jeannet, J. P., Wang, Y., Nathans, J., Dawid, I. B. & Varmus, H. E. (1997) *Science* **275,** 1652–1654.
20. Vinson, C. R. & Adler, P. N. (1987) *Nature (London)* **329,** 549–551.
21. Vinson, C. R., Conover, S. & Adler, P. N. (1989) *Nature (London)* **338,** 263–264.
22. Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S. & Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 802–806.
23. Sakata, H., Stahl, S. J., Taylor, W. G., Rosenberg, J. M., Sakaguchi, K., Wingfield, P. T. & Rubin, J. S. (1997) *J. Biol. Chem.* **272,** 9457–9463.
24. Finch, P. W., Rubin, J. S., Miki, T., Ron, D. & Aaronson, S. A. (1989) *Science* **245,** 752–755.
25. Kelley, M. J., Pech, M., Seuanez, H. N., Rubin, J. S., O'Brien, S. J. & Aaronson, S. A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9287–9291.
26. Zimonjic, D. B., Popescu, N. C., Matsui, T., Ito, M. & Chihara, K. (1994) *Cytogenet. Cell Genet.* **65,** 184–185.
27. Rubin, J. S., Chan, A. M., Bottaro, D. P., Burgess, W. H., Taylor, W. G., Cech, A. C., Hirschfield, D. W., Wong, J., Miki, T., Finch, P. W. & Aaronson, S. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 415–419.
28. McMahon, A. P. & Moon, R. T. (1989) *Development (Cambridge, U.K.)* **107,** Suppl., 161–167.
29. Chakrabarti, A., Matthews, G., Colman, A. & Dale, L. (1992) *Development (Cambridge, U.K.)* **115,** 355–369.
30. Wolda, S. L., Moody, C. J. & Moon, R. T. (1993) *Dev. Biol.* **155,** 46–57.
31. Smith, W. C. & Harland, R. M. (1991) *Cell* **67,** 753–765.
32. Turner, D. L. & Weintraub, H. (1994) *Genes Dev.* **8,** 1434–1447.
33. He, X., Saint-Jeannet, J. P., Woodgett, J. R., Varmus, H. E. & Dawid, I. B. (1995) *Nature (London)* **374,** 617–622.
34. Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8148.
35. Yang, B., Yang, B. L., Savani, R. C. & Turley, E. A. (1994) *EMBO J.* **13,** 286–296.
36. Zhao, Z., Lee, C. C., Baldini, A. & Caskey, C. T. (1995) *Genomics* **27,** 370–373.
37. Chan, S. D., Karpf, D. B., Fowlkes, M. E., Hooks, M., Bradley, M. S., Vuong, V., Bambino, T., Liu, M. Y., Arnaud, C. D., Strewler, G. J. & Nissenson, R. A. (1992) *J. Biol. Chem.* **267,** 25202–25207.
38. Rehn, M. & Pihlajaniemi, T. (1995) *J. Biol. Chem.* **270,** 4705–4711.
39. Hoang, B., Moos, M., Jr., Vukicevic, S. & Luyten, F. P. (1996) *J. Biol. Chem.* **271,** 26131–26137.
40. Shirozu, M., Tada, H., Tashiro, K., Nakamura, T., Lopez, N. D., Nazarea, M., Hamada, T., Sato, T., Nakano, T. & Honjo, T. (1996) *Genomics* **37,** 273–280.
41. Reichsman, F., Smith, L. & Cumberledge, S. (1996) *J. Cell Biol.* **135,** 819–827.
42. Kishimoto, T., Taga, T. & Akira, S. (1994) *Cell* **76,** 253–262.
43. Moon, R. T., Christian, J. L., Campbell, R. M., McGrew, L. L., DeMarais, A. A., Torres, M., Lai, C. J., Olson, D. J. & Kelly, G. M. (1993) *Development (Cambridge, U.K.) Suppl.*, 85–94.
44. Sokol, S., Christian, J. L., Moon, R. T. & Melton, D. A. (1991) *Cell* **67,** 741–752.
45. Dominguez, I., Itoh, K. & Sokol, S. Y. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8498–8502.
46. Pierce, S. B. & Kimelman, D. (1995) *Development (Cambridge, U.K.)* **121,** 755–765.
47. Rattner, A., Hsieh, J.-C., Smallwood, P. M., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. & Nathans, J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2859–2863.
48. Zecca, M., Basler, K. & Struhl, G. (1996) *Cell* **87,** 833–844.
49. Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M. & Ito, F. (1994) *Cell Adhes. Commun.* **1,** 295–305.
50. Huguet, E. L., Smith, K., Bicknell, R. & Harris, A. L. (1995) *J. Biol. Chem.* **270,** 12851–12856.
51. Leyns, L., Bouwmeester, T., Kim, S.-H., Piccolo, S. & De Robertis, E. M. (1997) *Cell* **88,** 747–756.
52. Wang, S., Krinks, M., Lin, K., Luyten, F. P. & Moos, M. J. (1997) *Cell* **88,** 757–766.

GENOMICS 37, 273–280 (1996)
ARTICLE NO. 0560

Characterization of Novel Secreted and Membrane Proteins Isolated by the Signal Sequence Trap Method

MICHIO SHIROZU,[1] HIDEAKI TADA,[1] KEI TASHIRO, TOMOYUKI NAKAMURA, NELSON D. LOPEZ, MARTINA NAZAREA, TSUNEYOSHI HAMADA, TOSHIHIKO SATO, TORU NAKANO,[2] AND TASUKU HONJO[3]

Department of Medical Chemistry, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606 Japan

Received March 11, 1996; accepted July 9, 1996

We recently described a method, called the signal sequence trap (SST) method, to clone cDNAs of secreted proteins and/or type I transmembrane proteins containing N-terminal signal sequences by using an epitope-tagging expression plasmid vector. In this paper we describe the summary of a large-scale screening of approximately 5900 clones of an SST cDNA library constructed from mouse bone marrow stromal cell line ST-2 cells. Of 26 positive clones obtained and sequenced, 11 clones appeared to contain authentic signal sequences. Five of the clones corresponded to the 5′ ends of the cDNA of known genes containing N-terminal signal sequences. The full-length cDNA clones of the 6 other unknown clones were isolated and sequenced. One clone, termed SDF3, encoded a mouse counterpart of human pigment epithelium-derived factor. Another clone, termed SDR1, had considerable homology with basigin, a member of the immunoglobulin superfamily. A third clone, termed SDF5, had partial homology with a *Drosophila* tissue polarity gene *frizzled* (*fz*) and its rat homologues, *fz-1* and *fz-2*. The other three clones had no significant homology with sequences in the databases. These results indicate that the SST method is effective and useful for the isolation of secreted and membrane proteins without knowledge of their functions. © 1996 Academic Press, Inc.

INTRODUCTION

The elucidation of molecular mechanisms for intercellular signaling and cell adhesion is essential for understanding the mechanism for the development and differentiation of multicellular organisms. Although a large number of molecules involved in signaling or adhesion have been cloned, there still remain many unknown molecules that are important for these functions. Most of the molecules involved in signaling or adhesion are secreted or membrane-anchored proteins, such as growth factors, hormones, neuropeptides, and their receptors or adhesion molecules. Many of these proteins contain hydrophobic signal sequences at the N-termini of the precursor forms. To establish a general cDNA cloning strategy for these secreted or membrane-anchored proteins, we developed a new cloning method for the isolation of cDNA fragments encoding N-terminal signal sequences (Tashiro *et al.*, 1993). cDNA fragments encoding signal sequences were detected by surface expression of the IL-2 receptor α chain (Tac) (Uchiyama *et al.*, 1981) as fusion proteins in transfected cells. Using this method, termed the signal sequence trap (SST) method, we have so far reported the isolation of cDNA for a member of the α-chemokines, SDF1α/β, and a growth factor receptor lymphotoxin β receptor, by the SST method (Tashiro *et al.*, 1993; Nakamura *et al.*, 1995), suggesting that this method may be widely useful.

The hematopoietic system is one of the best characterized organs for analyzing the mechanisms of mammalian cell growth and differentiation. Bone marrow stromal cell lines support hematopoiesis from bone marrow stem cells by secretion of cytokines and by interaction with cell-adhesion molecules. Many molecules involved in cell–cell interactions during lymphohematopoiesis have been cloned and are known to be expressed in bone marrow stromal cell lines (Metcalf, 1989; Paul *et al.*, 1991). However, there are still a number of unknown surface molecules involved in the early phases of hematopoiesis. We therefore performed a large-scale screening of cDNAs encoding secreted and transmembrane proteins from a bone marrow stromal cell line ST-2 (Nishikawa *et al.*, 1988) using the SST method. Isolated cDNAs will be expressed as proteins and their functions could be tested in an inductive system of lymphohematopoiesis from embryonic stem cells (Nakano *et al.*, 1994).

The nucleotide sequences reported in this paper have been deposited with the GenBank/EMBL Data Libraries under Accession Nos. SDF3 (Sdf3), D50460; SDF4 (Sdf4), D50461; SDF5 (Sdf5), D50462; SDR1 (Sdfr1), D50463; and SDR2 (Sdfr2), D50464.

[1] These authors contributed equally to this work.

[2] Present address: Department of Molecular Cell Biology, Research Institute for Microbial Diseases, Osaka University, Yamada-Oka, Suita, Osaka 565 Japan.

[3] To whom correspondence should be addressed at Department of Medical Chemistry, Kyoto University Faculty of Medicine, Yoshida, Sakyo-ku, Kyoto 606, Japan. Telephone: 81-75-753-4371. Fax: 81-75-753-4388.

0888-7543/96 $18.00

TABLE 1

Summary of the Trapped cDNA Clones of SST

Library	Clone	ORF in frame to ΔTAC	Homology: Known sequences	Homology: N-terminal SS	Hydrophobic peak	Stop codon upstream of initiation codon	Located at the 5′ end of full-length cDNA	Signal sequence[a]	Name of full-length clone
Library I	H1	+	+	+				T	Osteonectin
	H2	−						F	
	H3	+	−		Short			U	
	H4	+	+	−				F	Peptidyl-glycine α-amidating monooxygenase
	S1	+	−		+	−	+	T	SDR1
	S2	+	−		+	−	+	T	SDF3
	S3	+	−		+			U	
	S4	+	−		−			F	
	S6a	+	−		+	+		T	SDR2
	T1	+	−		+	−	+	T	SDF2
	T2	+	−		Short			U	
	T4	+	−		−			F	
	T5	+	+	+				T	Ribophorin 1
	T7	+	−		−			F	
	T8	+	−		Short			U	
	E6a	+	+	+				T	Protein disulfide isomerase
Library II	F7a	+	−		+	−	−	F	
	NT1	+	−		+			U	
	NT2	+	+	+				T	Fibronectin
	NT3	+	−		+	+	+	T	SDF4
	NT4	+	−		−			F	
	NT5	+	−		+	+	+	T	SDF5
	NT6	−						F	
	P5c	..						F	
	R5g	+	−		+	+	−	F	
	TA	+	+	+	+			T	Osteonectin

[a] T, true; F, false; U, undecided.

We will summarize the results of the large-scale screening of the SST library of ST-2 cells to examine the ratio of clones with the authentic signal sequence to artifacts and to test if this strategy is valid as a general cloning method for isolation of signal sequence-containing molecules. Ten clones with authentic signal sequences were obtained from approximately 5900 cDNA clones. The six full-length cDNA clones encoding novel proteins were isolated, sequenced, and characterized.

MATERIALS AND METHODS

Construction and screening of the SST library. Construction of the SST library was performed as described previously (Tashiro *et al.*, 1993) except that two sets of sonicated cDNA fragments with two size ranges (one between 300 and 600 bp and the other between 500 and 700 bp) were selected and used as inserts of the SST library. About 10,000 clones of the shorter insert cDNA library (library I) were plated on LB agar plates, and colony hybridization was performed with the probes of Gla (Ikeda *et al.*, 1991), JE (Rollins *et al.*, 1988), and SDF1 according to the conventional method (Sambrook *et al.*, 1989). Colonies that did not hybridize with any of the probes were picked up and spotted on 9-cm agar plates with a matrix format (7 rows by 7 lines) (Tashiro *et al.*, in press). Approximately 3500 preselected colonies were screened after 49 clones were pooled as described before (Tashiro *et al.*, 1993). The longer insert library (library II) was screened without preselection.

DNA sequencing. DNA sequencing was carried out using double-stranded templates by the dideoxy method using the *Taq* DyeDeoxy terminator cycle sequencing kit and an automated DNA sequencer (373A, Applied Biosystems). The sequence information was used for analysis of hydrophobicity and compared with sequences in the GenBank database for homology using BLAST and FASTA.

RNA extraction and Northern blot analysis. Crude RNAs of mouse tissues were extracted by using an RNA extraction kit (TRIZOL, GIBCO BRL), and poly(A)$^+$ RNAs were enriched by oligo(dT) latex (Oligotex(dT) Super, Takara). Two micrograms of mouse tissue poly(A)$^+$ RNA was electrophoresed through a 1.0% agarose gel and transferred to a nylon membrane (BIODYNE, Pall) in 10× SSC (1× SSC is 0.15 *M* NaCl, 15 m*M* sodium citrate) overnight, and cross-linked with a UV crosslinker (Funalinker, Funakoshi). The membrane was serially used for hybridization with the SDF3, SDF4, SDF5, SDR1, SDR2, and mouse β-actin probes radiolabeled by the random priming method (Sambrook *et al.*, 1989). The probes for the new genes were full-length cDNAs. Hybridization was performed according to a conventional method, and washing was performed under stringent conditions (Sambrook *et al.*, 1989). Autoradiograms were analyzed with a Bio-image analyzer (BAS 2000, Fuji Film).

RESULTS

cDNA Library Screening

In the previous study, a small-scale screening (approximately 600 clones) of the SST library with 300- to

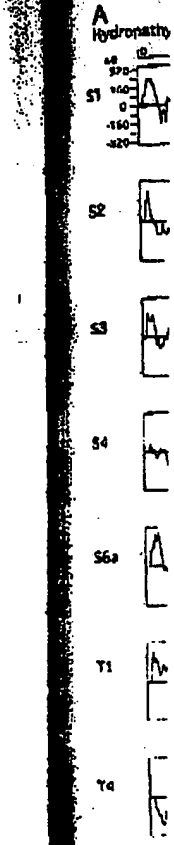


FIG. identics a hydro compute

600-bp isolati such a (a) elir izatior

Clone r
SDF1
SDF2
SDF3
SDF4
SDF5
SDR1
SDR2

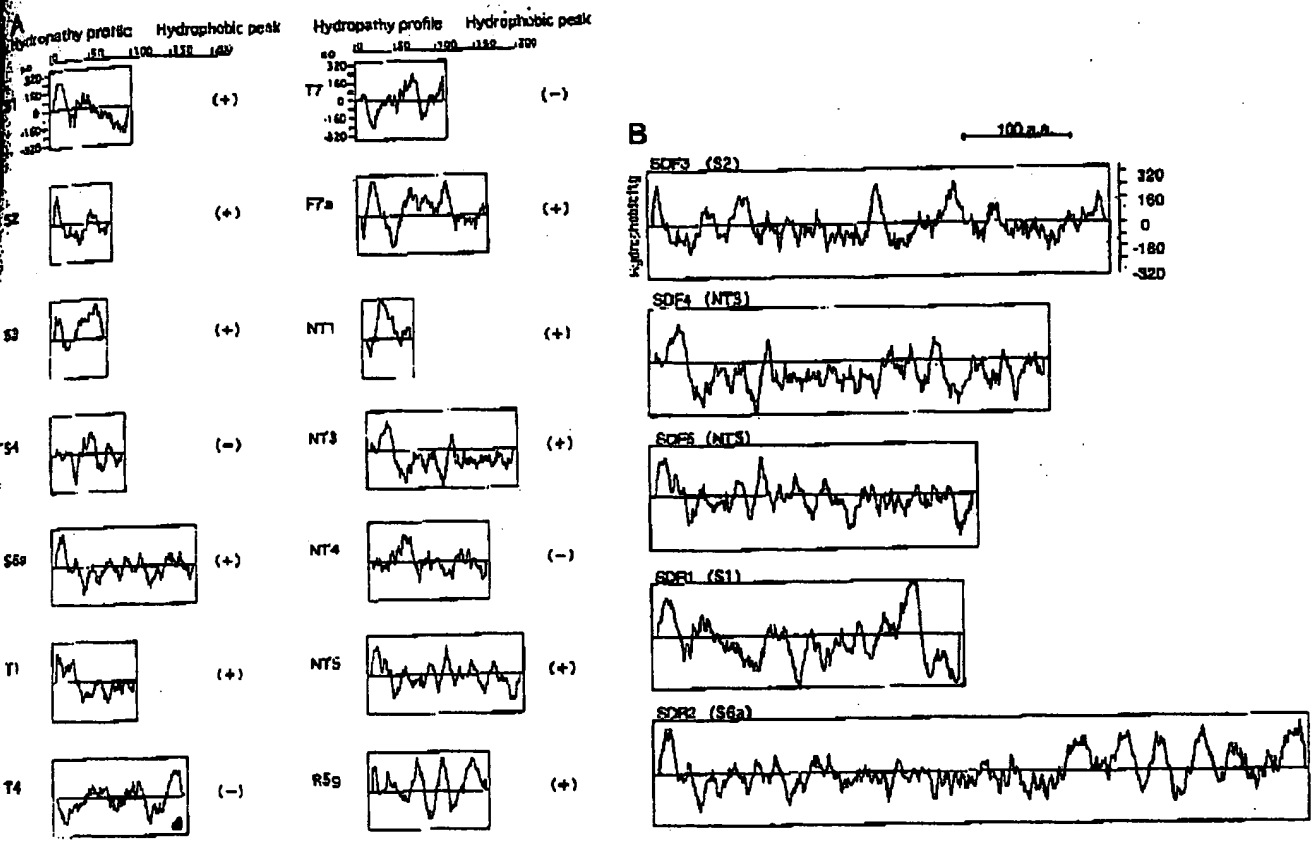


FIG. 1. (A) Hydropathy profiles of the signal sequence trapped clones. Hydropathy profiles of the 14 trapped clones excluding those identical to known genes or carrying short ORFs (fewer than 35 amino acid residues) are shown. (+) or (−) indicates a clone with or without a hydrophobic peak. (B) Hydropathy profiles of the full-length cDNA of newly identified clones. Hydropathy profiles were analyzed with a computer program based on Kyte and Doolittle (1982).

500-bp inserts (Tashiro *et al.*, 1993) led to the repeated isolation of three clones, Gla, JE, and SDF1. To exclude such abundant clones two strategies were introduced: (a) elimination of the abundant clones by colony hybridization (library I) and (b) construction of an SST library with longer inserts (library II), which should remove the three clones mentioned above because their coding sequences are less than 500 bp. The two strategies are complementary to some extent, because the former is tedious but does not exclude other shorter cDNA clones

TABLE 2

Summary of Characterization of the cDNA Clones Obtained by ST-2 SST

Clone name	cDNA length (kb)	ORF length (aa)	Homology	Sizes of mRNA (kb)	Tissue distribution of mRNA	References
SDF1	3.4 1.8	93 89	α-Chemokine	3.4, 1.8	Ubiquitous (peripheral blood leukocyte−)	Tashiro *et al.*, 1993
SDF2	1.0	211	Yeast Pmt1p and Pmt2p	1.0	Ubiquitous	Hamada *et al.*, in press
SDF3	1.4	417	Pigment epithelium-derived factor	1.6	Liver++, brain+, heart+, muscle+, spleen+, thymus+	This paper
SDF4	1.8	356	4 EF hands	5.0, 1.8	Ubiquitous	This paper
SDF5	1.8	295	*Drosophila frizzled* rat *frizzled-1* and *-2*	4.0, 2.2, 1.5	Heart+, kidney+, lung+, brain±, thymus±	This paper
SDR1	1.9	281	Ig superfamily basigin chicken HT-7	2.6, 2.1	2.1 kb, Ubiquitous 2.6 kb, brain-specific	This paper
SDR2	2.4	592		6.8, 2.6	Kidney+, liver±, spleen±, thymus±	This paper

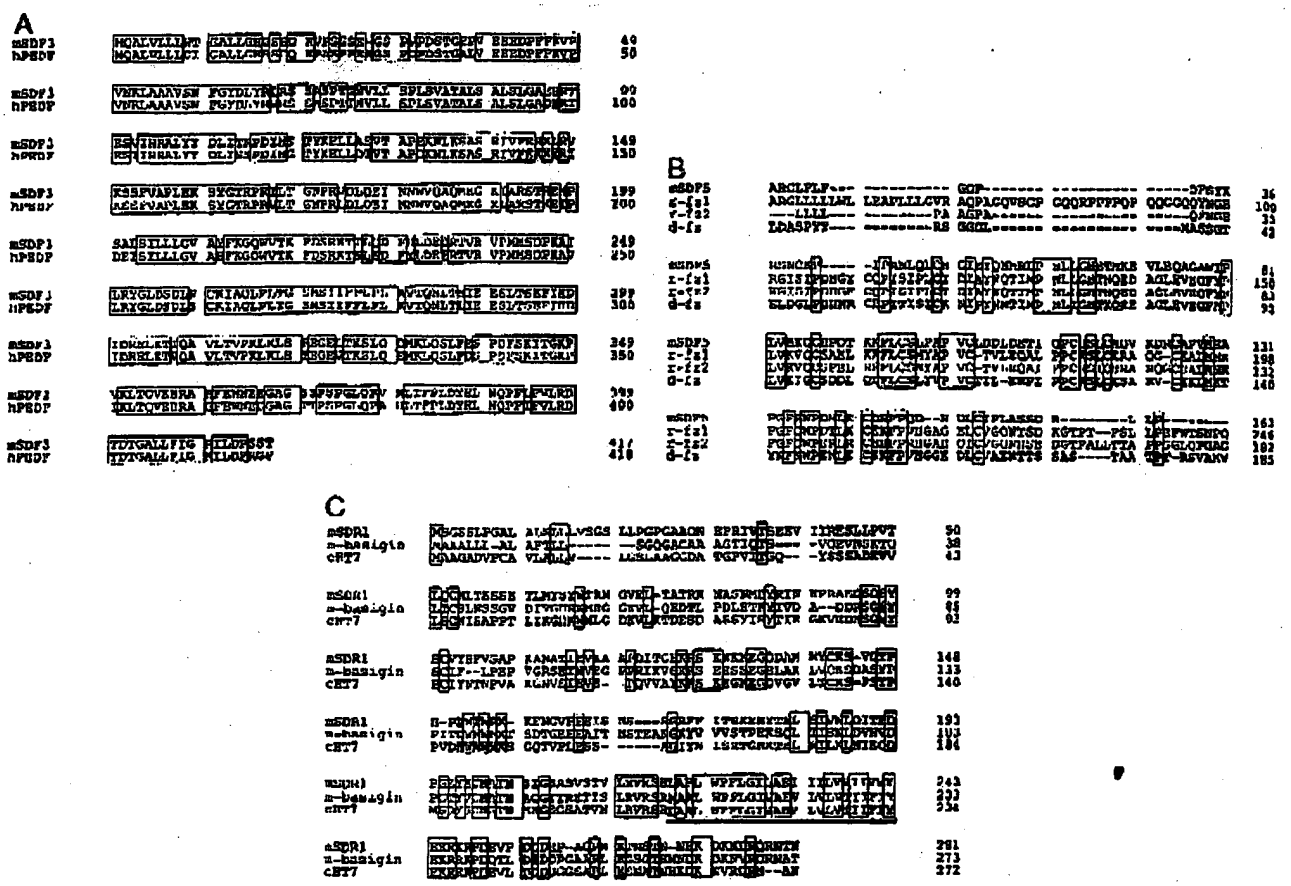


FIG. 2. Comparison of the amino acid sequences of SDF3, SDF5, and SDR1 with their homologues or related proteins. (A) Comparison of the amino acid sequences of SDF3 and human pigment epithelium-derived factor (PEDF). (B) Comparison of the amino acid sequences of SDF5 and *Drosophila fz* and its homologues, (rat *fz-1* and *fz-2*). (C) Comparison of the amino acid sequences of SDR1, mouse basigin, and chicken HT7. The putative transmembrane domains are underlined. The identical amino acid residues are indicated by open boxes. h, Human; m, mouse; r, rat; d, *Drosophila;* c, chicken.

while the latter might miss clones with open reading frames (ORFs) shorter than 500 bp.

About 8500 clones of library I were screened as described before (Tashiro *et al.*, 1993) and 15 positive clones were obtained. On the other hand, 11 positive clones were obtained by screening about 2400 clones of library II. The ratios of positive clones to artifact clones were similar between the two libraries and all of the 26 clones isolated will be characterized below.

Sequence Analysis of Positive SST cDNA Clones

The nucleotide and amino acid sequences of the inserts of the 26 positive clones were determined and compared with sequences in the GenBank database for homology. As summarized in Table 1, there were 6 clones identical to partial sequences of known genes as follows: H1 and TA, osteonectin precursor (Howe *et al.*, 1988); H4, peptidyl-glycine α-amidating monooxygenase precursor (Eipper *et al.*, 1992); T5, ribophorin 1 precursor (rough endoplasmic reticulum protein) (Behal *et al.*, 1990); E6a, protein disulfide isomerase precursor (rough endoplasmic reticulum protein) (Gong *et al.*, 1988); NT2, fibronectin precursor (Schwarzbauer *et al.*, 1987). Two signal sequence-trapped clones, H1 and TA, corresponded to the 5′ portions of osteonectin precursor cDNA, but the inserts of the two clones were not identical because they were derived from two distinct libraries. The hydropathy profiles of 14 clones are shown in Fig. 1A, whereas those of the other 12 clones identical to known genes or carrying no or very short ORFs (fewer than 35 amino acid residues) are excluded.

Structural examination of the 26 trapped cDNA clones did not allow us to conclude that the following 15 clones have authentic signal sequences. Three clones, H2, NT6, and P5c, had no ORFs in frame with the Tac cDNA sequence of the cloning vector. H4 was identical to part of a known gene, but it did not correspond to the N-terminal signal sequence (data not shown). S4, T4, T7, and NT4 had no obvious hydrophobic peaks following the putative first methionine residues. H3, T2, and T8 had very short ORFs (fewer than 35 aa) though their sequences had hydrophobic proper-

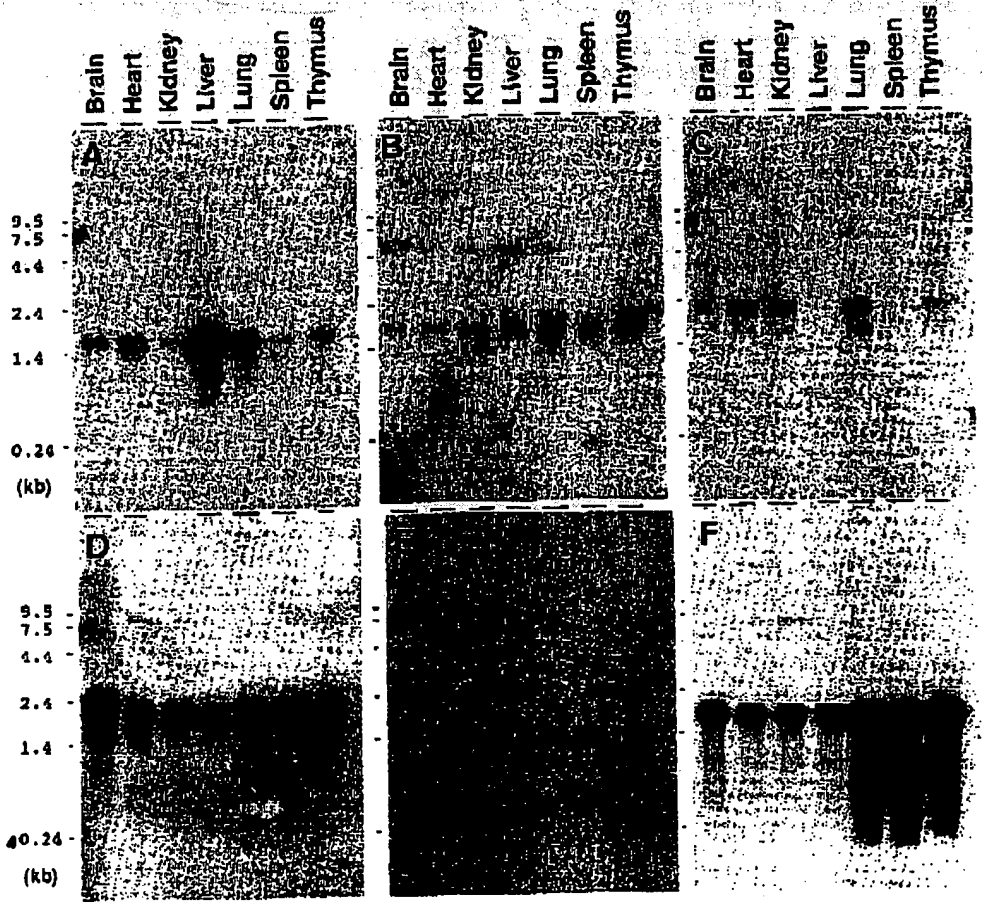


FIG. 3. Tissue distribution of SDF3, SDF4, SDF5, SDR1, and SDR2 mRNAs. The same Northern blot filter containing 2 μg of poly(A)$^+$ RNA from heart, brain, kidney, liver, lung, spleen, and thymus was hybridized serially with a DNA probe of SDF3 (A), SDF4 (B), SDF5 (C), SDR1 (D), SDR2 (E), or β-actin (F) cDNA. The filter was washed after each exposure. Size markers are indicated in kilobases.

ties (data not shown), and therefore it was very difficult to judge whether the sequences were authentic signal sequences or not. As S3 and NT1 had hydrophobic regions far from the first putative methionine residue (72 and 64 residues, respectively), we did not characterize them further although these hydrophobic sequences may serve as signal sequences. The trapped fragments of F7a and R5g did not locate at the 5′ ends of the full-length cDNA clones and these clones were not characterized further.

The following five known clones and six unknown clones were concluded to contain authentic signal sequences. Five clones, H1, T5, E6a, NT2, and TA, were identical to the N-terminal signal sequences of known genes (Table 1). The trapped fragments of six clones (S1, S2, S6a, T1, NT3, and NT5) were located at the 5′ ends of the full-length cDNA clones.

Sequence Analysis of Full-Length cDNAs of the Newly Identified Clones

Full-length cDNA of the six unknown clones with authentic signal sequences were isolated, sequenced, and characterized (Table 2). Detailed data of isolation and characterization of the SDF-2 clone were described elsewhere (Hamada *et al.*, in press). The hydropathy profiles of the deduced amino acid sequences were plotted (Fig. 1B). All the clones have the typical N-terminal signal sequences and none of them have KDEL signals for retention in the endoplasmic reticulum (Pelham *et al.*, 1989). A stromal cell-derived receptor (SDR) or stromal cell-derived factor (SDF) was named depending on the presence or absence, respectively, of a putative transmembrane region. SDR1 bears a clear hydrophobic region near the C-terminus and SDR2 has six hydrophobic domains at the C-terminus.

The sequence homology search was performed using GenBank and SWISS-PROT databases. SDF2 has significant homology to putative enzymic regions of yeast dolichyl phosphate-D-mannose: protein mannosyltransferase, Pmt1p, and Pmt2p (Hamada *et al.*, in press). SDF3 has remarkable homology to the human pigment epithelium-derived factor (PEDF) (Steele *et al.*, 1992) (Fig. 2A). There were 78 and 85% identities at the nucleotide and amino acid sequence levels, re-

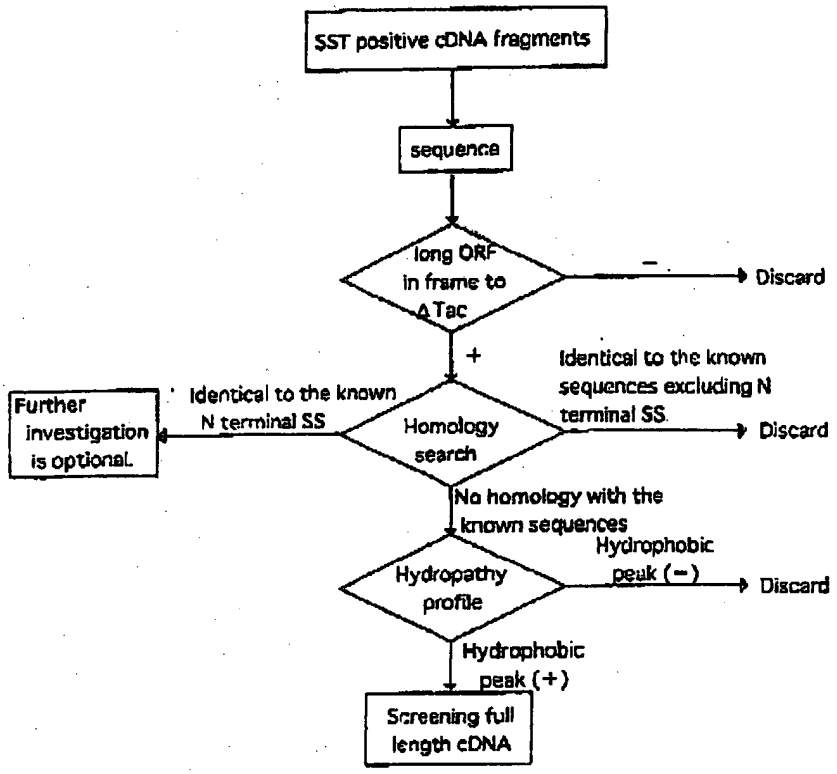


FIG. 4. The strategy for selection of SST positive cDNA fragments. SS, signal sequence.

spectively. This overall similarity strongly suggests that SDF3 is a mouse counterpart for the human PEDF. SDF4 has four EF hand motifs for calcium-binding activity (Kretsinger and Nockolds, 1973). SDF5 shows some homology to the extracellular domains of the *Drosophila* tissue polarity gene *frizzled* (Vinson *et al.*, 1989) and its rat homologues *fz-1* and *fz-2* (Chan *et al.*, 1992) (Fig. 2B), but its C-terminus has no homology with frizzled. SDR1 has a considerable homology to mouse basigin (Altruda *et al.*, 1989) and chicken HT7, members of the immunoglobulin superfamily (Fig. 2C). At the amino acid sequence level there is 38% identity between SDR1 and basigin, 38% identity between SDR1 and HT7, and 28% identity among the three proteins, while there is 45% identity between basigin and HT7. SDR2 shows no significant similarity to known genes.

Expression of mRNA of Novel Signal Sequence Trapped Clones

The tissue distribution of SDF3, SDF4, SDF5, SDR1, and SDR2 mRNAs was examined by Northern blot analysis (Fig. 3). SDF3, SDF4, SDR1, and SDR2 mRNAs were expressed in almost all the organs tested, although the expression levels were variable. Several observations are worth noting. SDF3 mRNA was strongly expressed in liver. SDF5 mRNA was expressed in brain, heart, kidney, lung, and thymus, but not in liver and spleen. A strong 2.1-kb transcript of the SDR1 gene was expressed ubiquitously but a 2.6-kb transcript was detected only in brain. SDR2 mRNA was relatively abundant in kidney.

DISCUSSION

In this report, we described a large-scale screening of two ST-2 SST libraries. Twenty-six positive clones were obtained from 5900 clones from the two libraries. Sequence analysis of the 26 clones revealed that 11 clones contained authentic signal sequences (true), 10 clones were classified as artifacts (false), and 5 clones were classified as undecided (undecided) (Table 1). Artifacts described here are either technical or strategic. The former sequences do not contain signal sequences at all, as judged by the absence of ORFs (H2, NT6, and P5c) or hydrophobic regions (H4, S4, T4, T7, and NT4). The latter sequences do not contain authentic N-terminal signal sequences but do have hydrophobic sequences in the middle of the full-length cDNA clones (F7a and R5g). Undecided clones contain atypical hydrophobic sequences: very short hydrophobic ORFs (H3, T2, and T8) and very long hydrophobic sequences (S3 and NT1). Given the frequencies of artifacts (38%) and novel genes (23%), we conclude that SST is an efficient method for isolating novel secreted or membrane-bound proteins.

After positive clones are screened by the SST method, further discrimination should be required to obtain novel cDNA clones encoding authentic N-terminal signal sequences (Fig. 4). The following strategy is empirically recommended. The first step is sequencing the trapped cDNA clones. It should be verified if the clones have a reasonably long (more than 35 residues) ORF in frame with the Tac sequence of the vector. If not, the clones should be abandoned as artifacts. The next step is a homology search using available databases of published genes and proteins. The clones identical to the known sequences should not be characterized further. The third test is selection by the hydropathy profile. Clones that have no hydrophobic peak at the N-terminus should be eliminated. In this way the clones that appear to contain authentic signal sequences are selected, and their full-length clones are isolated from a full-length cDNA library. The presence of an in-frame stop codon upstream of the first ATG codon is not mandatory because three unknown clones with authentic signal sequences (SDR1, SDF2, and SDF3) did not have upstream in-frame stop codons (see Table 1).

The biggest problem in a random screening approach is to determine the functions of isolated clones. Several strategies will facilitate the understanding of the functions of these molecules: patterns of tissue distribution, overexpression in appropriate cell lines, generation of antibodies against the expressed proteins, and gene targeting. Among the novel genes identified here, we gained some hints about the functions of SDF3 and SDR1 from their homology with known genes. SDF3 had remarkable homology to PEDF, suggesting that SDF3 is the mouse counterpart for human PEDF (Fig. 2A). PEDF is an extracellular neurotrophic agent and a member of the serine protease inhibitor family according to amino acid sequence similarity, but recombinant PEDF did not inhibit any known serine proteases (Becerra *et al.*, 1993). The tissue distribution of human PEDF mDNA had not yet been reported except for RPE cells (Tombran-Tink *et al.*, 1994), but mouse SDF3 mRNA was detected in all the organs tested, suggesting that SDF3 may have other functions in addition to neurotrophic activity. SDR1 belongs to the immunoglobulin superfamily and has considerable homology to mouse basigin and chicken HT7. The mouse basigin was first identified as a membrane protein (gp42) from a mouse fibroblast expression library by screening with a polyclonal antiserum (Altruda *et al.*, 1989). HT7 was identified as an antigen intensely expressed in the blood–brain barrier of the chicken and was closely related to basigin according to the amino acid similarity. As already indicated for basigin and HT7 (Miyauchi *et al.*, 1991), the transmembrane and cytoplasmic domains were highly conserved among the three molecules (Fig. 2C). The amino acid sequences of basigin and HT7 showed 63 and 49% identity, respectively, with that of SDR1. The functions of these molecules are not yet known. The brain-specific 2.6-kb transcript may be a splicing variant or a product of another related gene.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Science, and Culture of Japan. We are grateful to Dr. K. Shibahara for gifts of mouse tissue poly(A)$^+$ RNAs. We also thank Ms. H. Ohori, Ms. S. Okazaki, and Ms. E. Tadokoro for technical and secretarial assistance.

REFERENCES

Altruda, F., Cervella, P., Gaeta, M. L., Daniele, A., Giancotti, F., Turone, G., Stefanuto, G., and Silengo, L. (1989). Cloning of cDNA for a novel mouse membrane glycoprotein (gp42): Shared identity to histocompatibility antigens, immunoglobulines and neural-cell adhesion molecules. *Gene* 85: 445–452.

Becerra, S. P., Palmer, I., Kumar, A., Steele, F., Shiloach, J., Notario, V., and Chader, G. J. (1993). Overexpression of fetal human pigment epithelium-derived factor in *Escherichia coli*. *J. Biol. Chem.* 268: 23148–23156.

Behal, A., Prakash, K., D'Eustachio, P., Adesnik, M., Sabatini, D. D., and Kreibich, G. (1990). Structure and chromosomal location of the rat ribophorin I gene. *J. Biol. Chem.* 265: 8252–8258.

Chan, S. D. H., Karpf, D. B., Fowlkes, M. E., Hooks, M., Bradley, M. S., Vuong, V., Bambino, T., Liu, M. Y. C., Arnaud, C. D., Strewler, G. J., and Nissenson, R. A. (1992). Two homologs of the *Drosophila* polarity gene frizzled (fz) are widely expressed in mammalian tissues. *J. Biol. Chem.* 267: 25202–25207.

Eipper, B. A., Green, C. B. R., Campbell, T. A., Stoffers, D. A., Keutmann, H. T., Mains, R. E., and Ouafik, L. (1992). Alternative splicing and endoproteolytic processing generate tissue-specific forms of peptidylglycine alpha-amidating monooxygenase (PAM). *J. Biol. Chem.* 267: 4008–4015.

Gong, Q.-H., Fukuda, T., Parkison, C., and Cheng, S.-Y. (1988). Nucleotide sequence of a full-length cDNA clone encoding a mouse cellular thyroid hormone binding protein (p55) that is homologous to protein disulfide isomerase and the b-subunit of prolyl-4-hydroxylase. *Nucleic Acids Res.* 16: 1203.

Hamada, T., Tashiro, K., Tada, H., Inazawa, J., Shirozu, M., Shibahara, K., Nakamura, T., Nazarea, M., Nakano, T., and Honjo, T. (1996). Isolation and characterization of a novel secretory protein, stromal cell-derived factor-2 (SDF-2) using the signal sequence trap method. *Gene*, in press.

Howe, C. C., Overton, G. C., Sawicki, J., Solter, D., Stein, P., and Strickland, S. (1988). Expression of SPARC/osteonectin transcript in murine embryos and gonads. *Differentiation* 37: 20–25.

Ikeda, T., Yamaguchi, A., Icho, T., Tsuchida, N., and Yoshiki, S. (1991). cDNA and deduced amino acid sequence of mouse matrix bla protein: One of five glutamic acid residues potentially modified to gla is not conserved in the mouse sequence. *J. Bone Miner. Res.* 6: 1013–1017.

Kretsinger, R. H., and Nockolds, C. E. (1973). Carp muscle calcium-binding protein. II Structure determination and general description. *J. Biol. Chem.* 248: 3313–3326.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157: 105–132.

Metcalf, D. (1989). The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature* 339: 27–30.

Miyauchi, T., Masuzawa, Y., and Muramatsu, T. (1991). The Basigin group of the immunoglobuline superfamily: Complete conservation of a segment in and around transmembrane domains of human and mouse Basigin and chicken HT7 antigen. *J. Biochem.* 110: 770–774.

Nakamura, T., Tashiro, K., Nazarea, M., Nakano, T., Sasayama, S.,

and Honjo, T. (1995). The murine lymphotoxin-β receptor cDNA: Isolation by the signal sequence trap and chromosomal mapping. *Genomics* 30: 312–319.

Nakano, T., Kodama, H., and Honjo, T. (1994). *Science* 266: 1098–1101.

Nishikawa, S.-I., Ogawa, M., Nishikawa, S., Kunisada, T., and Kodama, H. (1988). B lymphopoiesis on stromal cell clone: Stromal cell clones acting on different stages of B cell differentiation. *Eur. J. Immunol.* 18: 1767–1771.

Paul, S. R., Yang, Y. C., Donahue, R. E., Goldring, S., and Williams, D. A. (1991). Stromal cell-associated hematopoiesis: Immortalization and characterization of a primate bone marrow-derived stromal cell line. *Blood* 77: 1728–1733.

Pelham, H. R. B. (1989). Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5: 1–23.

Rollins, B. J., Morrison, E. D., and Stiles, C. D. (1988). Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc. Natl. Acad. Sci. USA* 85: 3738–3742.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schwarzbauer, J. E., Patel, R. S., Fonda, D., and Hynes, R. O. (1987). Multiple sites of alternative splicing of the rat fibronectin gene transcript. *EMBO J.* 6: 2573–2580.

Steele, F. R., Chader, G. J., Johnson, L. V., and Tombran-Tink, J. (1992). Pigment epithelium-derived factor: Neurotrophic activity and identification as a member of the serine protease inhibitor gene family. *Proc. Natl. Acad. Sci. USA* 90: 1526–1530.

Tashiro, K., Nakano, T., and Honjo, T. (1996). "Methods in Molecular Biology." Humana Press, Totowa, NJ. In press.

Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., and Honjo, T. (1993). Signal sequence trap: A cloning strategy for secreted proteins and type I membrane proteins. *Science* 261: 600–603.

Tombran-Tink, J., Pawar, H., Swaroop, A., Rodriguez, I., and Chader, G. J. (1994). Localization of the gene for pigment epithelium-derived factor (PEDF) to chromosome 17p13.1 and expression in cultured human retinoblastoma cells. *Genomics* 19: 266–272.

Uchiyama, T., Broder, S., and Waldman, T. A. (1981). A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. *J. Immunol.* 126: 1393–1397.

Vinson, C. R., Conover, S., and Adler, P. N. (1989). A *Drosophila* tissue polarity locus encodes a protein containing seven potential transmembrane domains. *Nature* 338: 263–264.

617-876-5851

DEPARTMENT OF COMMERCE
UNITED STATES OF AMERICA

FACSIMILE COVER SHEET
PTO GROUP 1800
Official Fax Number (703) 305-3014

FROM: [redacted] Susan Ungar

ART UNIT: [redacted] 1642

SERIAL NO: _____

TO: Steve Lazar

COMPANY: Gov't 5288B

FAX NUMBER: _____

OF PAGES: 10 (including this page)

IF YOU DO NOT RECEIVE A LEGIBLE COPY OR IF YOU DO NOT RECEIVE ALL OF THE PAGES, PLEASE CALL THE EXAMINER AT (703) ~~305-7446~~ 305-2481, OR THE GROUP RECEPTIONIST AT (703) 308-0196.

THANK YOU.

```
RESULT    2
ID   O35297          PRELIMINARY;   PRT:   295 AA.
AC   O35297;
DT   01-JAN-1998 (TREMBLREL. 05, CREATED)
DT   01-JAN-1998 (TREMBLREL. 05, LAST SEQUENCE UPDATE)
DT   01-JAN-1998 (TREMBLREL. 05, LAST ANNOTATION UPDATE)
DE   SECRETED APOPTOSIS RELATED PROTEIN 1.
GN   SARP1.
OS   MUS MUSCULUS (MOUSE).
OC   EUKARYOTA; METAZOA; CHORDATA; VERTEBRATA; TETRAPODA; MAMMALIA;
OC   EUTHERIA; RODENTIA.
RN   [1]
RP   SEQUENCE FROM N.A.
RA   MELKONYAN H., CHANG W.C., SHAPIRO J.P., MAHADEVAPPA M.,
RA   FITZPATRIC P.A., KIEFER M.C., TOMEI D.L., UMANSKY S.R.;
RL   SUBMITTED (SEP-1997) TO EMBL/GENBANK/DDBJ DATA BANKS.
DR   EMBL; AF017989; G2415421; -.
SQ   SEQUENCE   295 AA;  33483 MW;  0E4C8A8E CRC32;

  Query Match          97.9%;  Score 2188;  DB 10;  Length 295;
  Best Local Similarity  97.6%;  Pred. No. 0.00e+00;
  Matches  288;  Conservative    4;  Mismatches    3;  Indels    0;  Gaps    0;

Db       1 MPRGPASLLLLVLASHCCLGSARGLFLFGQPDFSYKRSNCKPIPANLQLCHGIEYQNMRL 60
           | :||:|:|| |||||||||||||||||||||||||||||||||||||||||||||||||||
Qy       1 MLQGPGSLLLLFLASHCCLGSARGLFLFGQPDFSYKRSNCKPIPANLQLCHGIEYQNMRL 60

Db      61 PNLLGHETMKEVLEQAGAWIPLVMKQCHPDTKKFLCSLFAPVCLDDLDETIQPCHSLCVQ 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Qy      61 PNLLGHETMKEVLEQAGAWIPLVMKQCHPDTKKFLCSLFAPVCLDDLDETIQPCHSLCVQ 120

Db     121 VKDRCAPVESAFGFPWPDMLECDRFPQDNDLCIPLASSDHLLPATEEAPKVCEACKNKND 180
           |||||||:|||||||||||||||||||||||||||||||||||||||||||||||||||||||
Qy     121 VKDRCAPVMSAFGFPWPDMLECDRFPQDNDLCIPLASSDHLLPATEEAPKVCEACKNKND 180

Db     181 DDNDIMETLCKNDFALKIKVKEITYINRDTKIILETKSKTIYKLNGVSERDLKKSVLWLK 240
           ||||||||:|||||||||||||||||||||||||||||||||||||||||||||||||||||||
Qy     181 DDNDIMETLCKNDFALKIKVKEITYINRDTKIILETKSKTIYKLNGVSERDLKKSVLWLK 240

Db     241 DSLQCTCEEMNDINAPYLVMGQKQGGELVITSVKRWQKGQREFKRISRSIRKLQC 295
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Qy     241 DSLQCTCEEMNDINAPYLVMGQKQGGELVITSVKRWQKGQREFKRISRSIRKLQC 295


RESULT    3
ID   P97299          PRELIMINARY;   PRT:   295 AA.
AC   P97299;
DT   01-MAY-1997 (TREMBLREL. 03, CREATED)
DT   01-MAY-1997 (TREMBLREL. 03, LAST SEQUENCE UPDATE)
DT   01-JAN-1998 (TREMBLREL. 05, LAST ANNOTATION UPDATE)
DE   STROMAL CELL DERIVED FACTOR 5 (SDF5).
GN   SDF5.
OS   MUS MUSCULUS (MOUSE).
OC   EUKARYOTA; METAZOA; CHORDATA; VERTEBRATA; TETRAPODA; MAMMALIA;
OC   EUTHERIA; RODENTIA.
RN   [1]
RP   SEQUENCE FROM N.A.
RA   SHIROZU M., TADA H., JASHIRO K., NAYAMURA T., LOPEZ M.D.,
RA   NAZAREA M., HAMADA T., SATO T., NAKANO T., HONJO T.;
RL   GENOMICS 37:273-282(1996).
DR   EMBL; D50462; G1747302; -.
DR   MGD; MGI:108078; SDF5.
SQ   SEQUENCE   295 AA;  33501 MW;  329806C8 CRC32;

  Query Match          97.8%;  Score 2185;  DB 10;  Length 295;
  Best Local Similarity  97.6%;  Pred. No. 0.00e+00;
  Matches  288;  Conservative    4;  Mismatches    3;  Indels    0;  Gaps    0;

Db       1 MPRGPASLLLLVLASHCCLGSARGLFLFGQPDFSYKRSNCKPIPANLQLCHGIEYQNMRL 60
           | :||:|||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Qy       1 MLQGPGSLLLLFLASHCCLGSARGLFLFGQPDFSYKRSNCKPIPANLQLCHGIEYQNMRL 60

Db      61 PNLLGHETMKEVLEQAGAWIPLVMKQCHPDTKKFLCSLFAPVCLDDLDETIQPCHSLCVQ 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Qy      61 PNLLGHETMKEVLEQAGAWIPLVMKQCHPDTKKFLCSLFAPVCLDDLDETIQPCHSLCVQ 120

Db     121 VKDRCAPVESAFGFPWPDMLECDRFPQDNDLCIPLASSDHLLPATEEAPKVCEACKNKND 180
           |||||||:|||||||||||||||||||||||||||||||||||||||||||||||||||||||
Qy     121 VKDRCAPVMSAFGFPWPDMLECDRFPQDNDLCIPLASSDHLLPATEEAPKVCEACKNKND 180

Db     181 DDNDIMETLCKNDFALKIKVKEITYINRDTKIILETKSKTIYKLNGVSERDLKKSVLWLK 240
           ||||||||:|||||||||||||||||||||||||||||||||||||||||||||||||||||||
Qy     181 DDNDIMETLCKNDFALKIKVKEITYINRDTKIILETKSKTIYKLNGVSERDLKKSVLWLK 240

Db     241 DSLQCTCEEMNDINAPYLVMGQKQGGELVITSVKRWQKGQREFKRISRSIRKLQC 295
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Qy     241 DSLQCTCEEMNDINAPYLVMGQKQGGELVITSVKRWQKGQREFKRISRSIRKLQC 295


RESULT    4
ID   O14778          PRELIMINARY;   PRT:   206 AA.
AC   O14778;
DT   01-JAN-1998 (TREMBLREL. 05, CREATED)
DT   01-JAN-1998 (TREMBLREL. 05, LAST SEQUENCE UPDATE)
DT   01-JAN-1998 (TREMBLREL. 05, LAST ANNOTATION UPDATE)
DE   SECRETED APOPTOSIS RELATED PROTEIN 1 (FRAGMENT).
GN   SARP1.
OS   HOMO SAPIENS (HUMAN).
OC   EUKARYOTA; METAZOA; CHORDATA; VERTEBRATA; TETRAPODA; MAMMALIA;
OC   EUTHERIA; PRIMATES.
RN   [1]
RP   SEQUENCE FROM N.A.
RA   MELKONYAN H., CHANG W.C., SHAPIRO J.P., MAHADEVAPPA M., FITZPATRIC P.A.,
RA   KIEFER M.C., TOMEI D.L., UMANSKY S.R.;
RL   SUBMITTED (SEP-1997) TO EMBL/GENBANK/DDBJ DATA BANKS.
DR   EMBL; AF017986; G2415415; -.
FT   NON_TER      206     206
SQ   SEQUENCE   206 AA;  23215 MW;  B4659688 CRC32;

  Query Match          68.8%;  Score 1538;  DB 2;  Length 206;
  Best Local Similarity  98.6%;  Pred. No. 0.00e+00;
  Matches  205;  Conservative    0;  Mismatches    1;  Indels    2;  Gaps    1;

Db       1 MLQGPGSLLLLFLASHCCLGSARGLFLFGQPDFSYKRSNCKPIPANLQLCHGIEYQNMRL 60
           |||||||||||||||||||||||||||||||||||||.|||| ||||||||||||||||||
Qy       1 MLQGPGSLLLLFLASHCCLGSARGLFLFGQPDFSYKRSNCKPIPANLQLCHGIEYQNMRL 60

Db      61 PNLLGHETMKEVLEQAGAWIPLVMKQCHPDTKKFLCSLFAPVCLDDLDETIQPCHSLCVQ 120
           ||||||||||||||||||||||||||||||||||||||||||||:|||||||||||||||||
Qy      61 PNLLGHETMKEVLEQAGAWIPLVMKQCHPDTKKFLCSLFAPVCLDDLDETIQPCHSLCVQ 120

Db     121 VKDRCAPVNSAF--PWPDMLECDRFPQDNDLCIPLASSDHLLPATEEAPKVCEACKNKND 178
           |||||||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||
Qy     121 VKDRCAPVMSAFGFPWPDMLECDRFPQDNDLCIPLASSDHLLPATEEAPKVCEACKNKND 180

Db     179 DDSDIMETLCKNDFALKIKVKEITYIER 206
           ||||||||||||||||||||||||||||
Qy     181 DDSDIMETLCKNDFALKIKVKEITYIER 208


RESULT    5
ID   O14779          PRELIMINARY;   PRT:   314 AA.
AC   O14779;
DT   01-JAN-1998 (TREMBLREL. 05, CREATED)
DT   01-JAN-1998 (TREMBLREL. 05, LAST SEQUENCE UPDATE)
DT   01-JAN-1998 (TREMBLREL. 05, LAST ANNOTATION UPDATE)
DE   SECRETED APOPTOSIS RELATED PROTEIN 2.
GN   SARP2.
OS   HOMO SAPIENS (HUMAN).
OC   EUKARYOTA; METAZOA; CHORDATA; VERTEBRATA; TETRAPODA; MAMMALIA;
OC   EUTHERIA; PRIMATES.
RN   [1]
RP   SEQUENCE FROM N.A.
RC   TISSUE=HEART;
RA   MELKONYAN H., PROCHAZKA V., CHANG W.C., UMANSKY S.R.;
RL   SUBMITTED (SEP-1997) TO EMBL/GENBANK/DDBJ DATA BANKS.
```

JOURNAL OF BONE AND MINERAL RESEARCH
Volume 9, Number 11, 1994
Mary Ann Liebert, Inc., Publishers

Responsiveness of Clonal Limb Bud Cell Lines to Bone Morphogenetic Protein 2 Reveals a Sequential Relationship Between Cartilage and Bone Cell Phenotypes

VICKI ROSEN, JOHN NOVE, JEFFREY J. SONG, R. SCOTT THIES, KAREN COX, and JOHN M. WOZNEY

ABSTRACT

There is growing evidence to suggest that BMPs are among the signals necessary to create the embryonic skeleton, but how these regulatory molecules enter the pathways of embryonic bone formation remains to be defined. The earliest steps of endochondral bone formation, consisting of mesenchymal condensation and chondrogenesis, have been shown to result directly from BMP-2 action. To determine whether the transition from chondrogenesis to osteogenesis occurring later in endochondral bone formation is also the result of BMP activity, we tested the effects of BMP-2 on immortalized endochondral skeletal progenitor cells derived from mouse limb bud. The cell lines established by this process were found to fall into three general categories: undifferentiated skeletal progenitor cells, which in the presence of BMP-2 first express cartilage matrix proteins and then switch to production of bone matrix proteins; prechondroblast-like cells that constitutively express a subset of markers associated with chondrogenesis and, in the presence of BMP-2, shut off synthesis of these molecules and are induced to produce bone matrix molecules; and osteoblast-like cells that are not significantly affected by BMP-2 treatment. These data suggest that BMP-2 initiates the differentiation of limb bud cells into cells of both the cartilage and bone lineages in a sequential manner, making BMP-2 a potent regulator of skeletal cell differentiation.

INTRODUCTION

THE VERTEBRATE SKELETON is composed of a myriad of individual bony structures, each with a unique shape, size, and location. Although the outward appearance of these bones differs greatly, the cell types resident within each bone are the same. At the cellular level, formation of each individual bone follows one of two morphogenic paths: direct conversion of mesenchyme to bone tissue (intramembranous bone formation) or conversion of mesenchyme to cartilage, removal of the cartilage, and replacement by bone (endochondral bone formation). Why two paths for skeletal formation exist and the relationship between progenitor cells and regulatory molecules in these pathways have yet to be discovered.

There is growing evidence to suggest that bone morphogenetic proteins (BMPs) are among the signals necessary to create the embryonic skeleton, but how these regulatory molecules enter the pathways of embryonic bone formation remains to be defined. Individual BMPs, including BMP-2, have been localized by in situ hybridization in mouse embryos at sites undergoing skeletal formation.[1-3] In addition, mutant mice deficient in at least one of the BMPs, BMP-5, demonstrate impaired cartilage and bone formation during embryogenesis, as well as impaired fracture healing as adults.[4-6] In vitro, cells isolated from the developing skeleton are capable of responding to the BMPs, and BMPs can induce these embryonic cells to differentiate into osteoblast-like and chondroblast-like cell types.[7-12] These results correlate well with in vivo data on BMP activity: it is now well established that the soluble factors present in bone matrix that are responsible for bone-inductive activity are members of the BMP family.[13-19] These BMPs, when implanted in bone defects or in nonbony sites in adult animals,

Genetics Institute, Inc., Cambridge, Massachusetts.

initiate new bone formation through a sequence of events quite similar to embryonic endochondral bone formation. Undifferentiated mesenchymal cells present at the site of implantation convert into cartilage progenitor cells. These cells condense and produce abundant cartilage extracellular matrix, which is removed and replaced by bone.[20]

It is clear that mesenchymal condensation and the induction of chondrogenesis are a result of BMP action, but whether bone formation occurring at the end of the endochondral cascade can also be directly attributed to BMP activity is still speculative. To investigate the role of the BMPs in the differentiation of embryonic cells along the endochondral pathway, we used the limb bud as a source of endochondral skeletal progenitor cells and established stable clonal cell lines from 13 day postcoitus (13-dpc) mouse limbs.[10] In this report we show that BMP-2 initiates the differentiation of these cells into cells of both the cartilage and bone lineages, making BMP-2 a potent regulator of skeletal progenitor cell differentiation. We also begin to address the relationship between osteoprogenitor and chondroprogenitor cells as it may relate to endochondral bone formation.

MATERIALS AND METHODS

All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) in the presence of 10% heat-inactivated fetal calf serum (FCS; Hazelton, Denver, PA) and 4 mM L-glutamine unless otherwise noted. Recombinant human BMP-2 (BMP-2) was produced and purified as previously described.[21]

For creation of limb bud cell lines, embryos were removed from C57BL/6J mice at 13-dpc as determined by mating and measurement of the embryos upon retrieval. Whole fore- and hindlimb buds were collected and either used as whole buds or diced into four to six pieces. The whole buds or diced tissues were added to 100 mm dishes in the presence of DMEM containing 10% FCS and 8 μM Polybrene, together with a recombinant retroviral vector carrying the v-myc oncogene and a neomycin-selectable marker gene.[22] After incubation at 37°C for 5 h, the cultures were washed with phosphate-buffered saline (PBS), refed with DMEM and 10% FCS, and allowed to grow. After 1 week, G418 was added to the culture media in a stepwise manner (week 1, 250 μg/ml; week 2, 500 μg/ml; weeks 3–5, 1000 μg/ml) to select for cells that had incorporated the retroviral vector. We estimate that approximately 1% of the cells were neomycin resistant. Clonal cell lines were established from the neomycin-resistant cell pool using limiting dilution cloning, and these cell lines were subsequently characterized for cell phenotypes.

Assays

Alkaline phosphatase: Confluent cells, grown in DMEM with 10% FCS, were incubated for 24, 48, or 96 h in the presence or absence of test agent. After this treatment period, the cells were washed with PBS and then lysed by freeze thawing in water. Cell lysates were analyzed for alkaline phosphatase activity in 50 mM glycine, 0.05% Triton X-100, 4 mM $MgCl_2$, and 5 mM *p*-nitrophenol phosphate (Sigma, St. Louis, MO), pH 10.3, for 30 minutes at 37°C. Spectrophotometric absorbance at 405 nm was recorded for each sample and compared to *p*-nitrophenol standards to estimate alkaline phosphatase activity in the samples. Activities were normalized to cell protein, as measured by bicinchoninic acid assay (Pierce, Rockford, IL) using immunoglobulin G as standard.

Cyclic AMP production in response to parathyroid hormone (PTH): Confluent cells, grown in DMEM with 10% FCS, were preincubated with 1 mM 3-isobutyl-1-methylxanthine for 20 minutes at 37°C, followed by a 10 minute incubation with 400 ng/ml of PTH (fragment 1–34; Sigma, St. Louis, MO). The cells were lysed with trichloroacetic acid (TCA) and cellular cAMP determined in the cell lysates using a radioimmunoassay (RIA) kit (DuPont, Wilmington, DE).

Bone Gla protein (BGP) production: Confluent cells were treated with or without BMP-2 for 24–96 h. Conditioned medium from the last 24 h of treatment and cell layers were collected and assayed for the presence of immunoreactive BGP using a mouse BGP RIA assay kit (Biomedical Technologies, Inc., Stoughton, MA).

Radiolabeled sulfate incorporation: A total of 300,000 cells per well were seeded in 96-well dishes and incubated at room temperature for 2 h to form a micromass. The micromass cultures were treated for 96 h, with medium replacement every 24 h. For the last 24 h of treatment, medium was replaced with sulfate-free medium to which 10 μCi $^{35}SO_4$/ml was added. $^{35}SO_4$ incorporated into protein and cell layers was precipitated with 10% TCA at 4°C and quantified by liquid scintillation.

RNA preparation and analysis: Monolayer cells were grown to confluence in DMEM with 10% FCS and then changed to medium containing DMEM with 1% FCS ± BMP-2. Micromass cells were plated at 300,000 cells/well in 96-well dishes in DMEM containing 1% FCS. Total cellular RNA was isolated and prepared according to the method given in Sambrook et al.[23] RNA (10 μg/sample) was denatured and size separated on a 1% agarose gel containing 2.2 M formaldehyde and then transferred onto nitrocellulose (Schliecher and Scheull; 0.45 μm pore size) by capillary action.

Mouse proteoglycan core protein (C-PGN), collagens type I and II, and BGP cDNAs cloned at Genetics Institute were labeled for use as hybridization probes using a random priming kit (Pharmacia, Piscataway, NJ). Northern blots were prehybridized for 2 h and then hybridized overnight at 65°C in 5× saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, and 0.1 μg/ml of heat-denatured salmon sperm DNA. They were then washed once for 15 minutes at room temperature in 0.5× SSC and 0.1% SDS and then three times for 15 minutes each at 65°C in the same solution. Blots were exposed to Kodak XAR-5 film with intensifying screens at −80°C for 1–3 days. Quantitation of equal lane loading was done by staining blots with 0.04% methylene blue in 0.5 M sodium acetate (pH 5.2) for visualization of total cellular RNA as described by Sambrook et al.[23]

Histology and microscopy

Micromass cultures were fixed in 2% paraformaldehyde in PBS overnight at 4°C and then scraped from culture dishes and embedded in JB4 resin. Alkaline phosphatase was localized on 3

μm sections using the method of Troyer et al.[24] Monolayer cultures were stained for alkaline phosphatase activity using Sigma Kit 86-C.

Micromass cultures were also stained with alcian blue (pH 1.0) overnight after fixation for 30 minutes in 10% buffered formalin. Excess stain was rinsed with 0.1 M HCl.[25]

Photomicrographs were taken with a Zeiss Axiophot microscope.

RESULTS

Thirteen day postcoitus mouse limb bud cells were immortalized with a retroviral vector containing v-myc + neo and selected with G418 to obtain a cell pool, MLB13MYC, which was responsive to BMP-2 in a manner similar to primary MLB cells in preliminary studies.[10] Clonal cell lines were established from the MLB13MYC cell pool by a limiting cell dilution cloning technique. The resulting cell lines were screened for expression of chondroblast and osteoblast phenotypes using a panel of assays that included biochemical and molecular markers expressed by preosteoblasts and prechondroblasts, as well as markers that are more specifically associated with either bone or cartilage cells.[26–30]

As shown in Table 1, all the BMP-2-responsive cell lines expressed alkaline phosphatase, and many of these exhibited PTH sensitivity as well. These responses are consistent with both osteo- and chondroprogenitor cells but are not specific indicators of skeletal cell phenotypes. As an indication of the type of extracellular matrix (ECM) synthesized by MLB13MYC cell lines, we measured $^{35}SO_4$ incorporated into secreted protein and cell matrix by MLB13MYC clones, performed alcian blue staining on cell lines in micromass culture, and, using an RIA specific for BGP, monitored its production by MLB13MYC cells. If an individual cell line was alcian blue positive and incorporated $^{35}SO_4$ into secreted protein without synthesizing BGP, a protein associated with mature osteoblasts,[31] we preliminarily identified it as chondroblast-like. If an individual clone secreted measurable BGP, it was tentatively identified as osteoblast-like. These distinctions were useful in limiting the number of cell lines used for further studies. Before addition of BMP-2, 6 of the 24 cell lines exhibited a chondroblast-like ECM, and after BMP-2 treatment this number increased, so that 15 of the 24 lines studied produced a cartilaginous ECM as measured by our preliminary criteria. Relatively few of the cell lines synthesized BGP constitutively (4 of 24), but treatment with BMP-2 for at least 48 h strongly stimulated the production of BGP in 17 of the 24 lines. From this initial screen, we were able to define three types of cell lines: those that had no constitutive expression of chondroblast or osteoblast phenotype genes before BMP-2 treatment (skeletal progenitor cells), cell lines constitutively expressing low levels of one or more of the biochemical and molecular markers associated with chondroblast-like cells before BMP-2 addition (prechondroblast-like cells), and cell lines exhibiting a well-defined osteoblast phenotype without exposure to BMP-2 (osteoblast-like cells). We chose representatives of the first two phenotypes for further characterization.

MLB13MYC clone 14: A skeletal progenitor cell line

An example of an early skeletal progenitor cell clone that was derived from limb bud is MLB13MYC clone 14. These cells sequentially express morphologic and biochemical features, first of chondroblasts and then of osteoblasts, upon treatment with BMP-2. In monolayer cultures, MLB13MYC clone 14 cells appear stellate and become increasingly more cuboidal as they reach confluence (Plate 1A). Treatment of these cells with BMP-2 for 24 h induced measurable alkaline phosphatase activity (Plate 1B) that was significantly increased after 48 h of treatment and remained elevated throughout the remainder of the experiment (Fig. 1A). In addition, following 48 h of BMP-2 exposure, the MLB13MYC clone 14 cells were able to respond to PTH by increasing cAMP (250% increase after 48 h of treatment).

When we analyzed matrix protein synthesis and secretion, MLB13MYC clone 14 in monolayer cultures did not make detectable mRNA for C-PGN, collagen type II, or BGP without BMP-2 treatment. After 24 h of culture in the presence of BMP-2 (100 or 500 ng/ml), induction of mRNA for both C-PGN and collagen type II was apparent (Fig. 2). The mRNAs for C-PGN and collagen type II started to decline by 48 h and were nondetectable by day 4 of BMP-2 treatment. In contrast, mRNA for BGP and collagen type I, which appeared on days 2 and 4, respectively, in the BMP-2-treated cultures, continued to increase and remained elevated for the 8 day experimental period. Secretion of BGP protein by MLB13MYC clone 14 cells was not detected until 72 h after initiation of treatment with BMP-2 (data not shown).

To determine the duration of BMP-2 treatment needed to decrease expression of cartilage-associated matrix proteins and initiate expression of bone-associated proteins, we performed a time course experiment with monolayer cultures, treating cells for 1, 2, or 4 days, and then analyzed mRNAs at day 8. As seen in Fig. 3, treatment with BMP-2 for 24 h initiates production of mRNAs for C-PGN and collagen type II, but these are significantly decreased with increasing time in culture and are undetectable by the end of the 8 day period. BMP-2 treatment for 48 h appeared sufficient to cause a marked decrease in the levels of both collagen type II and C-PGN mRNAs and to induce synthesis of BGP mRNA by MLB13MYC clone 14 cells. The low levels of each of these mRNAs is probably the result of the ongoing switch process that starts during the 48 h treatment period. Continuous BMP-2 treatment for 4 days results in the switch from the production of collagen type II and C-PGN mRNAs to the production of BGP mRNA, which is maintained, although at a reduced level, at day 8. Continuous treatment of the cultures with 100 ng/ml of BMP-2 results in both the switch from chondroblast-like to osteoblast-like phenotype and maintenance of a high level of BGP mRNA.

In micromass cultures, MLB13MYC clone 14 cells showed a rounded morphology and, in the presence of BMP-2, synthesized significant amounts of alkaline phosphatase. By day 8 of BMP-2 treatment, the cells in micromass cultures showed an inner layer of hypertrophic cells surrounded by a distinct layer of flattened cells that stained intensely for alkaline phosphatase (Plate 2). When matrix proteins were anlayzed in micromass cultures of MLB13MYC clone 14, we observed the sequential induction of collagen type II and C-PGN mRNAs, followed by

TABLE 1. SUMMARY OF BONE AND CARTILAGE PHENOTYPE MARKER ASSAYS PERFORMED WITH ALL MLB13MYC CELL LINES[a]

Group	ALP	PTH cAMP	$^{35}SO_4$	BGP
−BMP-2	6/24	12/24	6/24	4/24
+BMP-2	18/24	12/24	15/24	17/24

[a]Values represent fraction of total clones that scored positively in these assays. ALP, alkaline phosphatase.

their decline and the induction of the mRNAs for BGP and collagen type I proteins (data not shown), a result similar to that seen when MLB13MYC clone 14 cells are maintained in monolayer cultures. We also used the micromass system to measure ECM production by MLB13MYC clone 14 cells. Although the amount of ECM deposited under these conditions was small, after 3 days of BMP-2 treatment there was an increase in sulfate incorporation into both secreted proteins and the cell layer (Table 2), suggesting that BMP-2 influences ECM production on the protein level.

MLB13MYC clone 17: A prechondroblast-like cell line

MLB13MYC clone 17 cells had very low but detectable amounts of mRNA for C-PGN and collagen type II when maintained in monolayer cultures (Fig. 4). The cells also displayed a constitutive ability to respond to PTH by increasing cAMP, and exposure of MLB13MYC clone 17 cells to BMP-2 for 48 h significantly enhanced their sensitivity to PTH by about 2-fold (data not shown). On the histologic level, the cells displayed little alkaline phosphatase activity without BMP-2, and treatment of confluent MLB13MYC clone 17 cells with BMP-2 for 24 h induced alkaline phosphatase activity to a low but significant level (Plate 3A and B). After 48 h of BMP-2 treatment, the induction of alkaline phosphatase activity was greatly increased, and a clear dose relationship was evident (Fig. 1B). With the highest dose of BMP-2 tested, we observed an 18-fold induction of alkaline phosphatase. Treatment of MLB13MYC clone 17 with BMP-2 for greater than 48 h did not significantly change alkaline phosphatase production from the level seen at 48 h. After 96 h of continuous BMP-2 treatment, alkaline phosphatase levels in MLB13MYC clone 17 cells remained elevated (Fig. 1B).

TABLE 2. $^{35}SO_4$ UPTAKE BY MLB13MYC CLONE 14 CELLS IN MICROMASS CULTURES[a]

BMP-2 (ng/ml)	Conditioned medium	Cell layer
0	633 ± 126	878 ± 94
1	449 ± 99	1,432 ± 572
10	733 ± 206	2,346 ± 236
100	4,868 ± 1321[b]	9,289 ± 2389[c]
1000	27,685 ± 11816[b]	12,215 ± 1800[c]

[a]Values are triplicate samples expressed as CPM/μG DNA and represent a typical experiment.
[b]Conditioned medium $n = 16 \pm$ standard error of the mean (SEM), $p < 0.0005$.
[c]Cell layer $n = 3 \pm$ SEM, $p < 0.05$.

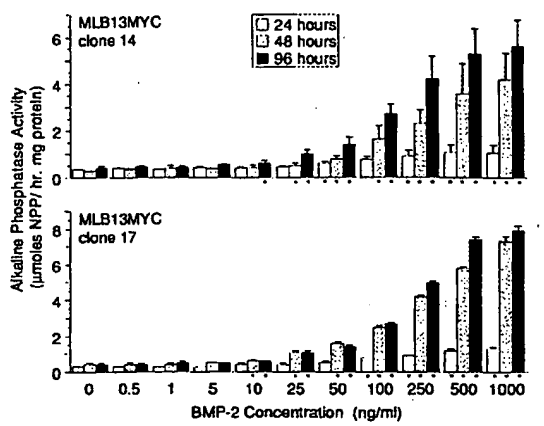


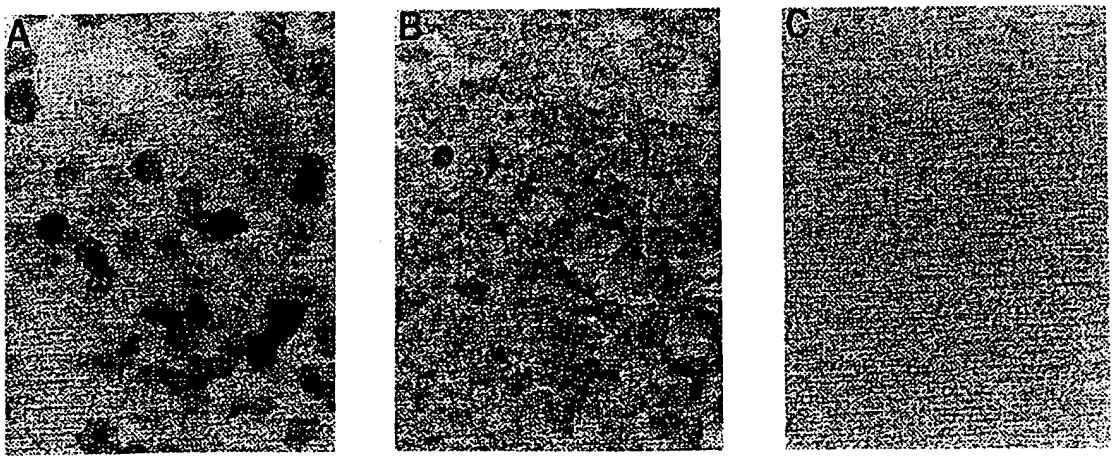
FIG. 1. Effect of BMP-2 on alkaline phosphatase activity in MLB13MYC clone 14 and MLB13MYC clone 17 cells as a function of BMP-2 dose and length of treatment. Cultures were plated at 20,000 cells per well in 96-well dishes and treated as shown. Results are expressed as the mean ± SEM of three experiments. Dots indicate significance, $p < 0.05$. (A) MLB13MYC clone 14; (B) MLB13MYC clone 17.

To examine the differentiation of MLB13MYC clone 17 cells treated with BMP-2, we performed northern analysis on mRNA from cells grown in monolayer and treated with 100 ng/ml of BMP-2 for 1–8 days. After 24 h of BMP-2 treatment, C-PGN mRNA present in untreated cells had decreased significantly and was undetectable at 48 h; a similar loss of collagen type II mRNA occurred 48 h after BMP-2 addition (Fig. 4). Within the same time period, the mRNAs for BGP and collagen type I, osteoblast-associated matrix proteins became evident in BMP-2-treated cells and were present for the remainder of the 8 day experimental period. We also measured BGP

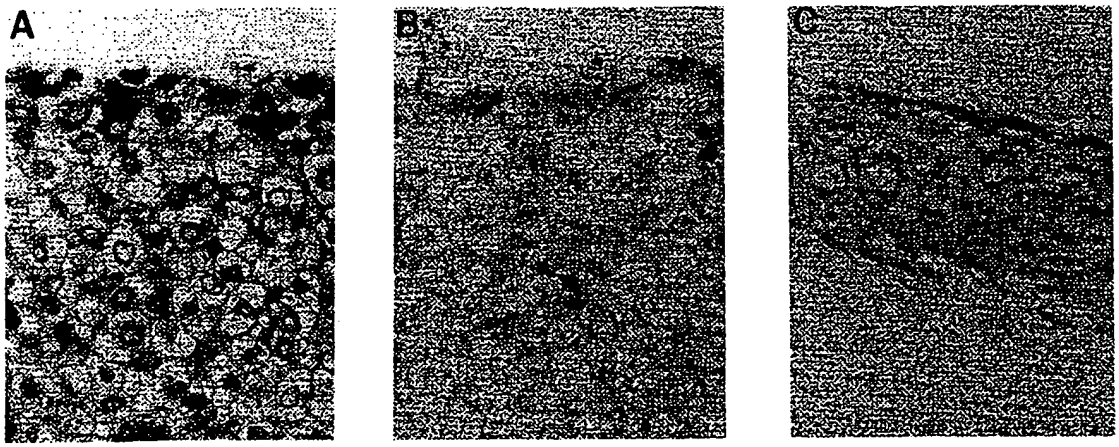
PLATE 1. MLB13MYC clone 14 cells in monolayer culture at confluence and stained for the presence of alkaline phosphatase after treatment with 100 ng/ml of BMP-2 for 24 (A) or 48 (B) h. Alkaline phosphatase is seen as a purple stain on few cells (A) at 24 h and on most cells (B) at 48 h but is absent from control cultures at 48 h (C). Original magnification: ×250.

PLATE 2. Photomicrographs of sections of MLB13MYC clone 14 cells in micromass cultures after 8 days of BMP-2 treatment (A, toluidine blue; B, alkaline phosphatase reactivity is red). The cells have a hypertrophic appearance in all BMP-2-treated cultures. An intense alkaline phosphatase stain (red) is seen in the outer layer of flattened, nonhypertrophic cells (C). Original magnification: ×500.

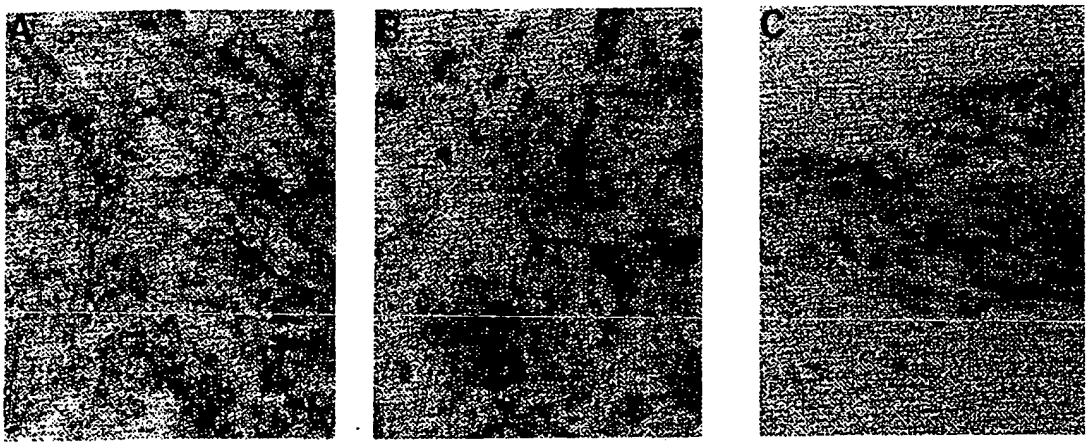
PLATE 3. MLB13MYC clone 17 cells in monolayer cultures in the presence of BMP-2 for 24 h (A, BMP-2-treated cultures; B, control cultures). Alkaline phosphatase, as indicated by purple staining, is seen in few of the cells in the absence of BMP-2 and in a majority of BMP-2-treated cells at 48 h. In histologic sections of micromass cultures of MLB13MYC clone 17 after 8 days of BMP-2 treatment, cells have a flattened morphology and do not appear hypertrophic (C). Alkaline phosphatase staining is red. Original magnification: A and B, ×250; C, ×500.



1



2



3

secretion by MLB13MYC clone 17 cultures and found accumulation of BGP in both the medium and cell layer by 96 h (data not shown).

When MLB13MYC clone 17 cells were plated in micromass and treated with BMP-2, in contrast to the histologic appearance of MLB13MYC clone 14 cells, no chondroblast-like cells were observed. Although the cells stained for alkaline phosphatase, the absence of alcian blue staining and their elongated appearance suggested that MLB13MYC clone 17 cells were no longer chondroblast-like (Plate 3C). These data support the idea that treatment of MLB13MYC clone 17 cells with BMP-2 results in the inhibition of a chondroblast-like phenotype and induction of an osteoblast-like phenotype. This phenotypic change was independent of any growth effects that BMPs may have, because MLB13MYC clone 17 showed no proliferative or antiproliferative responses to BMP-2 treatment (data not shown).

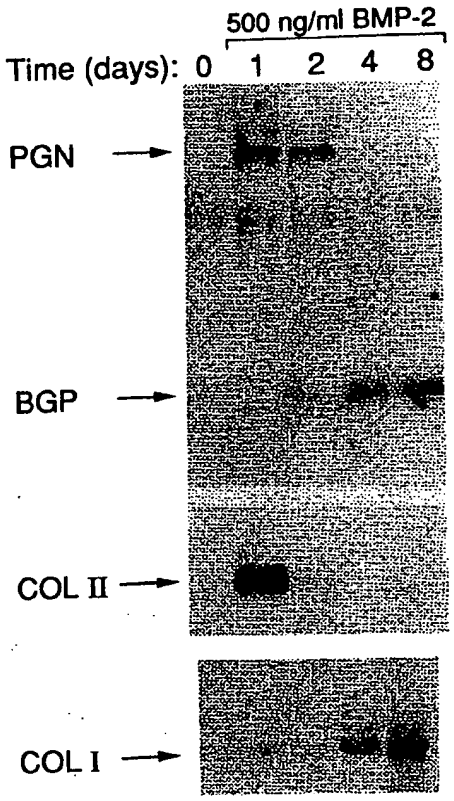


FIG. 2. Northern analysis of matrix protein mRNAs in MLB13MYC clone 14 cells in monolayer cultures. Cells were treated or not with 500 ng/ml of BMP-2 for the days indicated, and total RNA was collected and hybridized with cDNAs for cartilage-specific proteoglycan core protein (PGN), bone Gla protein (BGP), and collagens type I and II (COL I and COL II). RNA (10 μg) was loaded in each lane. Note that the faint bands that are present in all lanes in the COL I portion of the gel represent ribosomal RNA.

DISCUSSION

Embryonic limb is a well-recognized source of skeletal progenitor cells.[32,33] Immortalization of cells from 13-dpc mouse limb with v-myc oncogene has allowed us to establish a variety of clonal cell lines capable of expressing chondroblast-like and osteoblast-like phenotypes in vitro. The cell lines established by this process fall into three general categories: undifferentiated skeletal progenitor cells, which in the presence of BMP-2 first express cartilage matrix proteins and then switch to production of bone matrix proteins; prechondroblast-like cells that express a subset of markers associated with chondrogenesis and, in the presence of BMP-2, decrease the synthesis of these molecules and produce bone matrix molecules; and osteoblast-like cells that display a differentiated phenotype without BMP-2 treatment. These data are consistent with the role of BMPs as morphogens having direct actions on mesenchymal stem cells to initiate their differentiation into cells of the cartilage and bone lineages.

An early step in the endochondral bone formation process is the condensation of mesenchymal cells into discrete precartilaginous nodules.[34] In vivo, BMPs have been localized by in situ hybridization to presumptive skeletal areas before the onset of condensation, and in *short ear* mice, with a mutation in the BMP-5 locus, mesenchymal condensations during development are impaired.[2-6] These data suggest that BMPs are the natural inducers of mesenchymal condensation in vivo. In vitro, BMPs and BMP-related molecules have been shown to affect cell condensation. Recent data indicate that activin and transforming growth factor β, molecules related to BMPs, affect condensation in limb bud cells by altering expression of N-CAM.[35-37] BMP-7 has also been reported to increase expression of N-CAM in a glioblastoma-neuroblastoma hybrid.[38] Our own unpublished data showing BMP-2 regulation of N-CAM expression by MLB13MYC clone 14 support the idea that BMPs promote mesenchymal cell-progenitor cell condensations by affecting the level of N-CAM.

Once mesenchymal cell condensation has occurred, both early osteoblast-like and chondroblast-like cells can respond to PTH through increases in intracellular cAMP and also express high levels of alkaline phosphatase, an enzyme associated with matrix formation. All BMP-2 responding cells, 24 of the 28 cell lines we derived, constitutively expressed or could be induced to produce alkaline phosphatase and respond to PTH. MLB13MYC clone 14 and MLB13MYC clone 17 both responded to BMP-2 treatment with increases in PTH-induced cAMP and alkaline phosphatase activity.

The synthesis and secretion of an ECM rich in C-PGN and collagen type II is characteristic of the chondroblast phenotype. Low levels of collagen type II mRNA may be present before overt differentiation, but upregulation of these matrix macromolecules occurs as these cells mature.[33,39] Within 24 h of BMP-2 treatment, C-PGN and collagen type II mRNA were induced in both monolayer and micromass cultures of MLB13MYC clone 14. Continued treatment of both MLB13MYC clone 14 and MLB13MYC clone 17 with BMP-2 inhibited expression of the mRNAs for these cartilage matrix proteins, a pattern that has also been reported in primary cartilage cells undergoing hypertrophy.[40-42] Although we did not measure collagen type X, a marker of hypertrophic chondro-

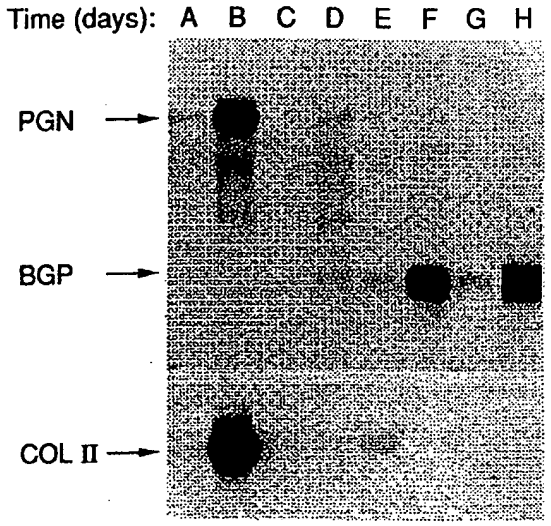


FIG. 3. Northern analysis of matrix protein mRNAs in MLB13MYC clone 14 cells in monolayer cultures treated or not with 100 ng/ml BMP-2 for 1, 2, 4, or 8 days in an 8 day experimental period. Total RNA was collected and hybridized with cDNAs for cartilage-specific proteoglycan core protein (PGN), bone Gla protein (BGP), and collagen type II (COL II). Lane designations: A, RNAs harvested before BMP-2 treatment; B, D, F, G, and H, RNAs harvested after 1 day (B), 2 days (D), 4 days (F), or 8 days (H) of continuous BMP-2 treatment; C, E, and G, RNAs harvested at the end of an 8 day period in which BMP-2 was present for the first day (C), first 2 days (E), or first 4 days (G). Lanes were loaded with 10 μg RNA.

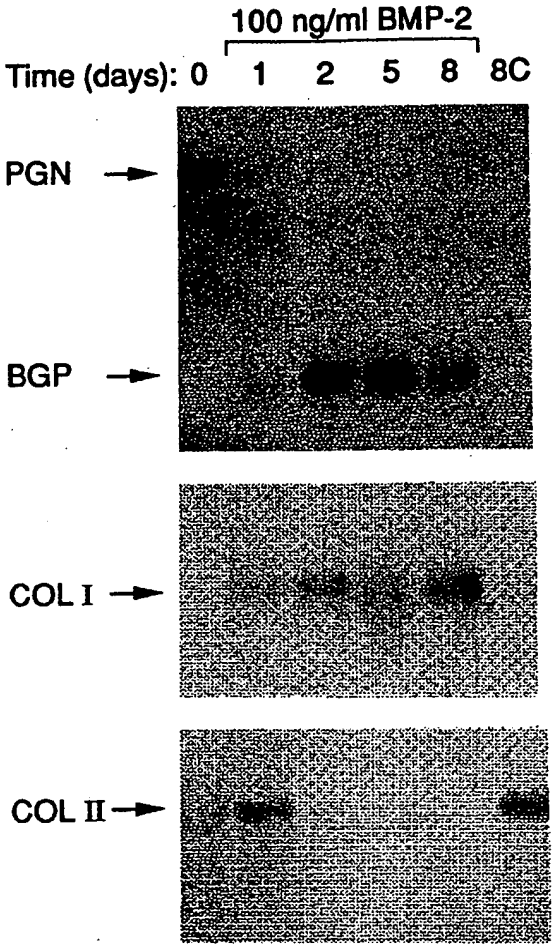


FIG. 4. Northern analysis of MLB13MYC clone 17 cells in monolayer cultures after 0, 1, 2, 5, or 8 days of BMP-2 treatment or after 8 days without BMP-2 treatment. Total RNA was collected at the end of the experimental period and hybridized with cDNAs for cartilage-specific proteoglycan core protein (PGN), bone Gla protein (BGP), and collagens type I and II (COL I and COL II). The 10 μg amounts of RNA were loaded in each lane.

cytes, histologic analysis of MLB13MYC clone 14 cells at 4 and 8 days of micromass cultures showed morphology consistent with a hypertrophic phenotype. This was despite the lack of ascorbic acid, glucocorticoids, retinoic acid, and high concentrations of FCS in our culture medium, all previously shown to be important for the elaboration of a hypertrophic phenotype in vitro.[40,41,43–45] It is possible that MLB13MYC clone 14 cells progress through a hypertrophic chondrocyte stage, and it will be interesting to characterize this progression under appropriate culture conditions in future studies.

V-myc immortalization has been reported to be well suited for cartilage progenitor cells, because the presence of the oncogene does not appear to affect cell differentiation during the early phases of chondrogenesis.[42,46,47] It may have effects on expression of a hypetrophic chondrocyte phenotype,[41,42] however, which would be in keeping with our observation of low levels of ECM production by MLB13MYC cells. The cell culture conditions used in these experiments, specifically the lack of ascorbic acid and glucocorticoids as additives to the culture medium, and also the use of greatly reduced serum concentrations (1%), have been shown to affect matrix deposition by chondrocytes in vitro, greatly reducing ECM deposited by these cells. From our studies we cannot establish whether the low levels of matrix deposition we observed are a result of the use of v-myc as an immortalizing agent or are caused by the incompatibility of our culture system with the requirements for ECM production. This question must be addressed in future experiments. Because our aim was to examine the effects of BMP-2 on the differentiation of mesenchymal cells into cartilage and bone, however, we believe our data indicate that BMP-2 induces the differentiation of skeletal progenitor cells along these pathways.

We do not believe that v-myc is responsible for the switch from a chondrocyte to an osteoblast-like phenotype observed when MLB13MYC clones 14 and 17 are treated with BMP-2, for a number of reasons. First, MLB13MYC cells maintained

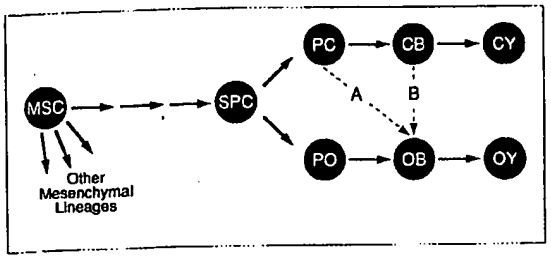


FIG. 5. Possible lineage diagram for differentiation of cells into mature cartilage and bone phenotypes. MSC, mesenchymal stem cell; SPC, skeletal progenitor cell; PC, prechondroblast; CB, chondroblast; CY, chondrocyte; PO, preosteoblast; OB, osteoblast; OY, osteocyte. We hypothesize that MLB13MYC clone 14 represents a SPC that in the presence of BMP-2 undergoes differentiation to PC and CB and then moves from the cartilage pathway to become an OB (broken arrow B is the switch step). MLB13MYC clone 17 represents a PC that in the presence of BMP-2 switches from the cartilage lineage to the bone cell lineage (broken arrow A is the switch step).

under similar culture conditions but without addition of BMP-2 do not differentiate into chondrocytes or osteoblasts. Second, treatment of our cultures with other agents known to affect chondroblast and osteoblast differentiation does not result in the elaboration of any osteoblast-like phenotype characteristics. Third, it has been reported that inhibition of endogenous c-myc expression by limb bud cells leads to enhanced chondrogenesis[48] and that v-myc immortalization prevents cells from displaying a mature chondrocyte phenotype,[41,42] findings that are inconsistent with v-myc immortalization leading to enhanced cellular differentiation by MLB13MYC cells. Instead, the need for continuous BMP-2 treatment for enhanced phenotype expression by MLB13MYC clone 14 cells suggests that v-myc may have a negative effect on phenotype expression in our system that is overcome by BMP-2. Last, recent work by Cancedda et al.[40,44] showed that primary hypertrophic chondrocytes, in the presence of retinoic acid (RA) and in monolayer culture, also downregulate type X collagen production and secrete BGP, a finding equated with continued differentiation of chondrocytes into osteoblasts in the absence of v-myc. Interestingly, Francis et al.[49] have shown that RA induces BMP-2 expression in developing chick limb in vivo, and it is possible that the presence of RA in the culture system used by Cancedda et al. may induce synthesis of BMP-2 protein, which would then be available to affect the further differentiation of hypertrophic chondrocytes into osteoblasts. These ideas remain to be proven, but our cell lines provide an interesting system in which to examine some of these interrelationships.

Our experimental evidence suggests that BMP-2 has a direct effect on two distinct stages of skeletal progenitor cells present in limb bud, revealing a sequential relationship between cartilage and bone cell phenotypes. Whether BMPs are the only stimuli that can elicit this response remains to be determined. Other growth factors known to be regulators of the cartilage cell phenotype, such as insulin-like growth factor I, fibroblast growth factor, and dexamethasone, did not induce expression of chondroblast phenotype by these cells but, in some cases, potentiated the expression of chondroblast properties in BMP-2-treated cells (data not shown). The response of MLB13MYC clones 14 and 17 was not unique, because 10 of the 24 cell lines created from 13-dpc limb buds had the ability to produce both cartilage-specific and bone-specific proteins when treated with BMP-2.

We propose that in vivo during embryonic bone formation, a morphogen, such as BMP-2, first induces the expression of a chondroblast-like phenotype. We further speculate that cartilage then remains as cartilage unless it is subject to another inductive signal, possibly local synthesis of BMPs or other, yet to be identified, molecules (see Fig. 5). Previous in vivo studies have suggested that phenotypically discernible cartilage cells transdifferentiate into bone cells, but the difficulties inherent in systems in vivo, notably heterogeneity of cell populations, have left doubt about chondroblast to osteoblast transitions.[50–54] Analysis of clonal cell populations that originate from a single primary chondrocyte have suggested transdifferentiation does occur. Our ability to use clonal cell lines providing homogeneous cell populations offers distinct advantages. By documenting the differentiation of MLB13MYC cells on a molecular and biochemical level, we have provided additional specific evidence for the sequential differentiation of cartilage cells to bone cell phenotypes, data consistent with reports of the ability of osteoprogenitor cells to express chondroblast-like phenotype features in vitro.[15,55,56] The availability of morphogenic molecules, such as BMP-2, allows us to control the differentiation of progenitor cells to osteoblasts and chondroblasts in both settings in vivo and in vitro. Given the large number of BMP family members and related proteins discovered to date, we speculate that each possesses distinct as well as overlapping activities that influence skeletal cell differentiation and morphogenesis.

ACKNOWLEDGMENTS

The authors thank Dr. M.E. Holtrop for her editing skills, J. Capparella, D. McQuaid, and L. Kurtzberg for their technical contributions, and P. Caires for preparing this manuscript.

REFERENCES

1. Jones MC, Lyons KM, Hogan BLM 1991 Involvement of bone morphogenetic protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. Development **111**:531–543.
2. Lyons KM, Pelton RW, Hogan BLM 1990 Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein (BMP-2a). Development **190**:833–844.
3. Lyons KM, Pelton RW, Hogan BLM 1989 Patterns of expression of murine Vgr-1 and BMP-2a suggest transforming growth factor β-like genes coordinately regulate aspects of embryonic development. Genes Dev **3**:1657–1668.
4. Green MC 1958 Effects of the *short ear* gene in the mouse on cartilage formation in healing bone fractures. J Exp Zool **137**:75–88.
5. Green EL, Green MC 1942 The development of three manifestations of the *short ear* gene in the mouse. J Morphol **70**:1–19.
6. Kingsley DM, Bland AE, Grubber JM, Marker PC, Russell LB, Copeland NG, Jenkins NA 1992 The mouse *short ear* skeletal

morphogenesis locus is associated with defects in a bone morphogenetic member of the TGF-β superfamily. Cell 71:399–410.
7. Carrington JL, Chen P, Yanagishita M, Reddi AH 1991 Osteogenin (bone morphogenetic protein-3) stimulates cartilage formation by chick limb bud cells in vitro. Dev Biol 146:406–415.
8. Chen P, Carrington JL, Hammond RG, Reddi AH 1991 Stimulation of chondrogenesis in limb bud mesoderm cells by recombinant human bone morphogenetic protein 2b (BMP-2b) and modulation by transforming growth factor-β_1 and β_2. Exp Cell Res 195:509–515.
9. Chen TL, Bates RL, Dudley A, Hammond RG Jr, Amento EP 1991 Bone morphogenetic protein 2b stimulation of growth and osteogenic phenotypes in rat osteoblast-like cells: Comparison with TGF-β_1. J Bone Miner Res 6:1387–1393.
10. Rosen V, Capparella J, McQuaid D, Cox K, Thies RS, Song JJ, Wozney JM 1993 Development of immortalized cells derived from 13dpc mouse limb buds as a system to study the effects of rhBMP-2 on limb bud cell differentiation. In: Bernfeld M (ed) Limb Development and Regeneration. Wiley-Liss, New York, pp. 305–315.
11. Vukicevic S, Luyten FP, Reddi AH 1989 Stimulation of the expression of osteogenic and chondrogenic phenotypes in vitro by osteogenin. Proc Natl Acad Sci USA 86:8793–8797.
12. Yamaguchi A, Katagiri T, Ikeda GT, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda T, Yoshiki S 1991 Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113:681–687.
13. Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM 1990 Identification of transforming growth factor-β family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci USA 87:9843–9847.
14. Cook SD, Baffes GC, Sampath TK, Rueger DC 1992 Healing of large segmental defect using recombinant osteogenic protein (rhOP-1). Trans Orthop Res Soc 17:581.
15. Hanumonds RG Jr, Schwall R, Dudley A, Berkemeire L, Lai C, Lee H, Cunningham N, Reddi AH, Wood WI, Mason AJ 1991 Bone inducing activity of mature BMP-2b produced from hybrid BMP-2a/2b precursor. Mol Endocrinol 5:49–155.
16. Ozkaynzk E, Rueger DC, Drier EA, Corbett C, Ridge RJ, Sampath TK, Oppermann H 1990 OP-1 cDNA encodes an osteogenic protein in the TGF-β family. EMBO J 9:2085–2093.
17. Urist MR 1965 Bone formation by autoinduction. Science 150:893–899.
18. Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel D, Hewick RM, Kerns K, LaPan P, Luxenberg DP, McQuaid D, Moutsatsos IM, Nove J, Wozney JM 1990 Recombinant human bone morphogenetic protein induces bone formation. Proc Natl Acad Sci USA 87:2220–2224.
19. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA 1988 Novel regulators of bone formation: Molecular clones and activities. Science 242:1528–1534.
20. Reddi AH 1981 Cell biology and biochemistry of endochondral bone development. Collagen 1:209–226.
21. Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V 1992 Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology 130:1318–1324.
22. Williams DA, Rosenblatt MF, Beier DR, Cone RD 1988 Generation of murine stromal cell lines supporting hematopoietic stem cell proliferation by use of recombinant retrovirus vectors encoding simian virus 40 large T antigen. Mol. Cell Biol 8:3864–3871.
23. Sambrook J, Fritsch EF, Maniatis T 1989 Cloning—A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.
24. Troyer H, Fisher OT, Rosenquist TH 1977 Alkaline phosphatase localization in plastic sections. Histochemistry 50:251–259.
25. Lev R, Spicer SS 1964 Specific staining of sulfate groups with alcian blue at low pH. J Histochem Cytochem 12:38–41.
26. Benya PD, Padilla SR, Nimni ME 1978 Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell 15:1313–1321.
27. Chase LR, Aurbach GD 1970 The effect of parathyroid hormone on the concentration of adenosine 3′,5′-monophosphate in skeletal tissue in vitro. J Biol Chem 245:1520–1526.
28. Nishimoto SK, Price PA 1980 Secretion of the vitamin K dependent protein of bone by rat osteosarcoma cells. J Biol Chem 255:6579–6583.
29. Robison R 1923 The possible significance of hexosephosphonic esters in ossification. Biochem J 17:286–293.
30. Solursh M, Meier S 1973 A conditioned medium (CM) factor produced by chondrocytes that promotes their own differentiation. Dev Biol 30:279–289.
31. Lian JB, Gundberg CM 1988 Osteocalcin: Biochemical consideration and clinical applications. Clin Orthop 226:267–291.
32. Ahrens PB, Solursh M, Reiter RS 1977 Stage-related capacity for limb chondrogenesis in cell culture. Dev Biol 60:69–82.
33. Kosher RA, Gay SW, Kamanitz JR, Kulyk WM, Rodgers BJ, Sai S, Tanaka T, Tanzer ML 1986 Cartilage proteoglycan core protein gene expression during limb cartilage differentiation. Dev Biol 118:112–117.
34. Thorogood PV, Hinchliffe JR 1975 An analysis of the condensation process during chondrogenesis in the embryonic chick hind limb. J Embryol Exp Morphol 33:581–606.
35. Jiang T-X, Yi JR, Ying S-Y, Chuong CM 1993 Activin enhances chondrogenesis of limb bud cells: Stimulation of precartilaginous mesenchymal condensations and expression of NCAM. Dev Biol 155:545–552.
36. Roubin R, Deagostini-Bazin H, Hirsch M-R, Goridis C 1990 Modulation of NCAM expression by transforming growth factor-β, serum, and autocrine factors. J Cell Biol 111:673–684.
37. Widelitz RB, Jing T-X, Murrary BA, Chuong C-M 1993 Adhesion molecules in skeletogenesis. II. Neural cell adhesion molecules (NCAM) mediate precartilaginous mesenchymal condensations and enhance chondrogenesis. J Cell Physiol 156:399–411.
38. Perides G, Rueger DC, Charness ME 1992 Induction of the neural cell adhesion molecule and neuronal aggregation by osteogenic protein-1. Proc Natl Acad Sci USA 89:10326–10329.
39. Wood A, Ashurst DE, Corbett A, Thorogood P 1991 The transient expression of type II collagen of tissue interfaces during mammalian craniofacial development. Development 111:955–968.
40. Castagnola P, Dozin B, Moro G, Cancedda R 1988 Changes in the expression of collagen genes show two stages in chondrocyte differentiation in vitro. J Cell Biol 106:461–467.
41. Iwamoto M, Shapiro IM, Yagami K, Boskey AL, Leboy PS, Adams SL, Pacifici M 1993 Retinoic acid induces rapid mineralization and expression of mineralization-related genes in chondrocytes. Exp Cell Res 207:413–420.
42. Quarto R, Doum B, Tacchetti C, Robino G, Zenke M, Campanile G, Cancedda R 1992 Constitute *myc* expression impairs hypertrophy and calcification in cartilage. Dev Biol 149:168–176.
43. Cancedda FD, Gentili C, Manduca P, Cancedda R 1992 Hypertrophic chondrocytes undergo further differentiation in culture. J Cell Biol 117:427–435.
44. Gentili C, Bianco P, Neri M, Malpeli M, Campanile G, Castagnola P, Cancedda R, Cancedda FD 1993 Cell proliferation, extracellular matrix mineralization, and ovotransferrin transient expression during in vitro differentiation of chick hypertrophic chondrocytes into osteoblast-like cells. J Cell Biol 122:709–712.
45. Grigoriadis AE, Heersche JNM, Aubin JE 1988 Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: Effect of dexamethasone. J Cell Biol 106:2139–2151.

46. Alema S, Tato F, Boettinger D 1985 *Myc* and *src* oncogenes have complementary effects on cell proliferation and expression of specific extracellular matrix components in definitive chondroblasts. Mol Cell Biol 5:538–544.
47. Horton WE Jr, Cleveland J, Rapp U, Nemuth G, Bolander M, Doege K, Yamada Y, Hassell JR 1988 An established rat cell line expressing chondrocyte properties. Exp Cell Res 178:457–468.
48. Motoyama J, Eto K 1994 Antisense c-*myc* oligonucleotide promotes chondrogenesis and enhances RA responsiveness of mouse limb mesenchymal cells in vitro. FEBS Lett 338:323–325.
49. Francis PH, Richardson MK, Brickell PM, Tickle C 1994 Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb. Development 120:209–218.
50. Richman JM, Diewert VM 1988 The fate of Menkel's cartilage chondrocytes in ocular culture. Dev Biol 129:48–60.
51. Roach HI 1992 Transdifferentiation of hypertrophic chondrocytes and cells capable of producing a mineralized bone matrix. Bone Miner 19:1–20.
52. Strauss PG, Closs EI, Schmidt J, Erfle V 1990 Gene expression during osteogenic differentiation in mandibular condyles in vitro. J Cell Biol 110:1369–1378.
53. Thesingh CW, Groot CG, Wassendar AM 1991 Transdifferentiation of hypertrophic chondrocytes into osteoblasts in murine fetal metatarsal bones, induced by cocultured cerebrum. Bone Miner 12:25–40.
54. Yoshikawa C, Yagi T 1988 Electron microscope observations on the fate of hypertrophic chondrocytes in condylar cartilage of rat mandible. J Craniofac Genet Dev Biol 8:253–264.
55. Bernier SM, Desjardins J, Sullivan AK, Goltzman D 1992 Establishment of an osseous cell line from fetal rat calvaria using an immunocytolytic method of cell selection: Characterization of the cell line and of derived clones. J Cell Physiol 145:274–285.
56. Grigoriadis AE, Heersche JNM, Aubin JE 1990 Continouously growing bipotential myogenic, adipogenic and chondrogenic subclones isolated from multipotential RCH3.1 clonal cell line. Dev Biol 142:313–318.

Address reprint requests to:
V. Rosen
Genetics Institute, Inc.
87 Cambridge Park Drive
Cambridge, MA 02140

Received in original form November 30, 1993; in revised form May 26, 1994; accepted May 30, 1994.

0013-7227/01/$03.00/0
Printed in U.S.A.

Endocrinology 142(9):4026–4039

Differential Regulation of the Two Principal Runx2/Cbfa1 N-Terminal Isoforms in Response to Bone Morphogenetic Protein-2 during Development of the Osteoblast Phenotype

CHAITALI BANERJEE, AMJAD JAVED, JE-YONG CHOI, JACK GREEN, VICKI ROSEN, ANDRÉ J. VAN WIJNEN, JANET L. STEIN, JANE B. LIAN, AND GARY S. STEIN

Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655; and Genetics Institute (V.R.), Cambridge, Massachusetts 02140

Cbfa1/Runx2 is a transcription factor essential for bone formation and osteoblast differentiation. Two major N-terminal isoforms of Cbfa1, designated type I/p56 (PEBP2aA1, starting with the sequence MRIPV) and type II/p57 (*til-1*, starting with the sequence MASNS), each regulated by distinct promoters, are known. Here, we show that the type I transcript is constitutively expressed in nonosseous mesenchymal tissues and in osteoblast progenitor cells. Cbfa1 type I isoform expression does not change with the differentiation status of the cells. In contrast, the type II transcript is increased during differentiation of primary osteoblasts and is induced in osteoprogenitors and in premyoblast C2C12 cells in response to bone morphogenetic protein-2. The functional equivalence of the two isoforms in activation and repression of bone-specific genes indicates overlapping functional roles. The presence of the ubiquitous type I isoform in nonosseous cells and before bone morphogenetic protein-2 induced expression of the type II isoform suggests a regulatory role for Cbfa1 type I in early stages of mesenchymal cell development, whereas type II is necessary for osteogenesis and maintenance of the osteoblast phenotype. Our data indicate that Cbfa1 function is regulated by transcription, cellular protein levels, and DNA binding activity during osteoblast differentiation. Taken together, our studies suggest that developmental timing and cell type-specific expression of type I and type II Cbfa isoforms, and not necessarily molecular properties or sequences that reside in the N-terminus of Cbfa1, are the principal determinants of the osteogenic activity of Cbfa1. (*Endocrinology* 142: 4026–4039, 2001)

THE RUNX/CBFA/AML/PEBPαA family of transcription factors includes three distinct genes that encode proteins with crucial roles in the regulation of cell fate decision and transcriptional control of critical genes for cellular differentiation and development (reviewed in Refs. 1 and 2). Several key studies have established that *Cbfa1* is required for *in vivo* bone formation (3–5) as well as maturation of hypertrophic chondrocytes (6, 7) and osteoblast differentiation (3, 8, 9). There is a complete lack of intramembranous and endochondral bone formation in *Cbfa1* null mice (3), and haploinsufficiency of this gene results in cleidocranial dysplasia (CCD), a dominantly inherited developmental disorder of bone (4, 5). The loss of bone formation is attributed to maturational arrest of the osteoblast differentiation process (3, 8, 9). *Cbfa1* is expressed in mesenchymal condensations of developing bones during embryogenesis (3, 5), and the mRNA has been shown to increase in osteogenic tissues (8–11).

In the past few years several isoforms for each of the three Cbfa transcription factors have been identified. A shared property of the Cbfa genes is that expression is regulated by at least two distinct promoters that generate two N-terminal isoforms (11–13). Additional isoforms arise as a result of alternative splicing, exon skipping, as well as deletions and frameshift mutations in the N-terminal, C-terminal, and internal regions of the gene (11, 14). Cbfa1 isoform structure and expression have been studied in rat, mouse, and human (9; 11, 13, 14) and in the context of CCD phenotype (15, 16) where patient genotypes reveal perturbation of the *Cbfa1* gene structure. The first *Cbfa1* isoform identified, PEBP2aA1 (17) and recently described as the type I isoform (18), is a 513-amino acid protein (designated p56/type I) that initiates in exon 2 at the sequence MRIPV. It was initially shown to be expressed in T cells and Ha-*ras*-transformed fibroblasts (17, 19) and thymus (20) and was detected in other nonosseous (21), chondrogenic (7, 22), and osteoblast cell lines (8, 11, 18). The second major isoform, *til-1* (14) (designated p57 or type II isoform), initiates in exon 1 at the sequence MASNS (11, 14, 23) and is only 15 amino acids longer than the p56/type I isoform. Forced expression of these isoforms modulates transcription of skeletal genes (18, 24–26), indicating that both proteins are functionally active in osteoblasts and hypertrophic chondrocytes. While attention has focused on the functional activities of these isoforms, their expression in relation to osteoblast maturation and differentiation under control of osteo-inductive factors remains to be addressed.

Abbreviations: ALP, Alkaline phosphatase; BMP, bone morphogenetic protein; CCD, cleidocranial dysplasia; CSK, cytoskeletal; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mALP, mouse alkaline phosphatase; OBSC, osteoblast-specific complex; OC, osteocalcin; poly(A)$^+$, polyadenylated; ROB, rat osteoblasts; TGFβ-RI, TGFβ type I receptor.

Members of the family of bone morphogenetic proteins, BMP-2 and BMP-4/-7, mediate the commitment of undifferentiated mesenchymal progenitor cells to the skeletal lineage (27–29) and are potent inducers of *Cbfa1* transcription (9, 18, 21, 30). Thus, the early events of osteogenesis, regulated by BMP-2, are closely linked to Cbfa1 induction of the genes involved in bone formation and osteoblast differentiation.

To increase understanding of the role of Cbfa1 in regulating osteogenesis, we examined the expression of Cbfa1 isoforms at different stages of osteoblast development. We also determined the BMP-2 responsiveness of the two major N-terminal isoforms in primary rat calvarial osteoblasts, mouse MC3T3 preosteoblasts, and cells that represent earlier stages of osteoprogenitors. Here we show that the type I transcript is constitutively expressed in nonosseous mesenchymal tissues and during osteoblast differentiation. However, expression of the type II transcript is regulated during osteoblast differentiation and is induced by BMP-2. Our studies provide novel insights into the regulation of Cbfa1 activity in relation to the development of skeletal lineage cells.

Materials and Methods

Cell culture

Normal diploid osteoblasts obtained from 21-d-old fetal rat calvariae were isolated and maintained as previously described (31). Primary cell cultures were established from postnatal mouse lung, liver, muscle, and skin tissues after 5- and 15-min sequential digestions with collagenase P (Roche Molecular Biochemicals, Mannheim, Germany), plating cells from the second digest at a density of 0.5×10^6/100-mm dish. MC3T3-E1 cells were maintained in αMEM supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA). MLB13MYC clone 14 and MLB13MYC clone 17 cell lines were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (32). Cells at passage 12 were changed to DMEM with 1% FBS when treated with 100 ng/ml BMP-2 (Genetics Institute, Cambridge, MA). C2C12 cells were maintained in DMEM (33) supplemented with 5% FBS and treated with 300 ng/ml BMP-2 when required. Charcoal-stripped serum was prepared for BMP-2-treated cells by the addition of 5% activated charcoal to FBS at 4 C overnight, followed by filter sterilization.

Plasmids and analysis of promoter activity

Several promoter-reporter constructs were used for transient transfection assays as previously described (25). Rat osteosarcoma ROS 17/2.8 cells and nonosseous HeLa cells plated on six-well plates were transiently transfected with 2.5 μg/well of either the 0.6-kb chick bone sialoprotein promoter-*chloramphenicol acetyltransferase* (CAT; a gift from Dr. L. Gerstenfeld, Musculoskeletal Research Laboratory, Boston University Medical Center, Boston, MA) (34), the 1.6-kb TGFβ type I receptor (TGF-βRI) promoter-*Luc* (gift from Dr. M. Centrella, Department of Surgery, Yale University School of Medicine, New Haven, CT) (35), or the 1.1-kb rat osteocalcin (OC) (36) promoter-*CAT*. The reporter constructs were cotransfected with 750 ng/well expression plasmid containing cDNAs of either PEBP2αA1 (type I; gift from Dr. Yoshiaki Ito, Department of Viral Oncology, Kyoto University, Kyoto, Japan) (17) or *til-1*(type II; gift from Dr. James Neil, Department of Veterinary Pathology, University of Glasgow, Glasgow, UK) (14) using Superfect transfection reagent (Life Technologies, Inc.). CAT activity was measured 24–36 h after transfection, and transfection efficiency was normalized by the luciferase activity of the internal control plasmid Rous sarcoma virus-*luciferase* (100 ng/well). Representative results of three independent studies are shown. Data shown are the mean ± SD (n = 9).

Protein-DNA interaction analysis

Nuclear extracts were prepared from proliferating (d 2 or 3), differentiated (d 14) and mineralized (d 20 or 23) primary rat osteoblasts as previously described (37) using 0.45 M KCl for extraction. For mouse MC3T3-E1 nuclear extracts, cells were collected on d 7, 10, 16, and 22. MLB13MYC clone 14 and MLB13MYC clone 17 cells [control and 48-h BMP-2-treated (100 ng/ml)] were collected on d 7, C2C12 cells [control and 48 h BMP-2-treated (300 ng/ml)] were collected on d 5. EMSAs were performed using conditions previously described (8). Four micrograms of nuclear extracts were incubated with 1 μg of the nonspecific competitor poly(dI-dC)(dI-dC) (Pharmacia Biotech, Piscataway, NJ) and 10 fmol ^{32}P end-labeled, double stranded Cbfa binding consensus oligonucleotide (5′-CGAGTATTGTGGTTAATACG-3′). Protein-DNA complexes were resolved in 4% nondenaturing polyacrylamide gels using Tris-glycine-EDTA buffer. Antibody supershift experiments contained polyclonal antiserum raised against a C-terminal peptide of Cbfa1 (38) or preimmune serum (control). Gels were dried and exposed to Kodak films (Eastman Kodak Co., New Haven, CT) at −70 C for 6–12 h.

Western blot analysis

Nuclear extracts (30 μg/lane) were resolved in 10% SDS-PAGE and electroblotted (using a semidry electroblotter; Owl Scientific Plastics, Cambridge, MA) onto nitrocellulose membranes (0.2 μm, Protran, Schleicher & Schuell, Inc., Keene, NH) according to the manufacturer's specifications. Western blot analyses were performed as previously described (8). Based on our data and those of others (35, 39–41), it is not possible to electrophoretically separate Runx2/Cbfa1 isoforms, type I (p56) and type II (p57), nor has it been possible to generate antibodies that can discriminate between these two proteins. Membranes were incubated at a 1:100 or 1:150 dilution of antibody in Tris-buffered saline containing 1% BSA. IgG-fractionated rabbit polyclonal antibody specific for Cbfa1 (38) or a mouse monoclonal antibody (16) was used in these studies. Membranes were incubated with secondary antibody for 45 min, followed by chemiluminescent detection using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's specifications. Membranes were exposed for 10 sec to 5 min to Amersham Pharmacia Biotech Hyperfilm for detection of signals. Lamin B antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Northern blot analysis

Total cellular RNA was isolated using TRIzol (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's specifications. Polyadenylated [poly(A)$^+$] RNA was isolated using an mRNA isolation kit (Roche, Indianapolis, IN). Ten micrograms of total RNA or 2 μg poly(A)$^+$ RNA/lane were separated in a 1% agarose-formaldehyde gel, transferred onto Zetaprobe membrane (Bio-Rad Laboratories, Inc., Hercules, CA), and hybridized to probes specific for Cbfa1 exon 1 (which detects the N-terminal region of type II isoform; GenBank accession no. AF155361), a full-length cDNA, or a 266-bp *Bam*HI-*Nco*I fragment of PEBP2aA1 (GenBank accession no. D14636) that is common to all isoforms. Hybridization was performed as previously described (42) in the presence of buffer containing 50% formamide at 42 C, and the blots were washed extensively in buffer containing 1 × SSC (standard saline citrate) and 0.1% SDS at 55 C. Data were analyzed after overnight exposure using a Storm 840 PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Ethidium bromide staining of the gels was used to assess equal loading of samples.

RT and PCR

RT was performed on total RNA using Moloney murine leukemia virus reverse transcriptase (Roche Molecular Biochemicals) as specified by the manufacturer. Total RNA (1–2 μg) was incubated at 37 C for 60 min in the presence of deoxy-NTPs, ribonuclease inhibitor (Promega Corp., Madison, WI), oligo(deoxythymidine)$_{15}$ primers (Promega Corp.), and reverse transcriptase. For PCR, *Taq* polymerase (Promega Corp.) was used in reactions containing cDNA from the RT reactions, deoxy-NTPs, 1 μM each of forward and reverse primers, and 1 mM $MgCl_2$. The forward primer for the type I isoform (p56/PEBP2αA1) corresponds to the N-terminal end of the published cDNA sequence of this isoform (GenBank accession no. D14636). The forward primer for the type II (p57/til-1) isoform corresponds to sequences upstream of the translational start site of til-1. The

reverse primer is common to both isoforms. PCR amplification primers for Cbfa1 and all other genes are listed in Table 1.

PCR was performed using a thermal controller, PTC-100 (MJ Research, Inc., Watertown, MA) under the following conditions: 95 C for 5 min, 94 C for 30 sec, 60 C for 30 sec, and 72 C for 30 sec, 30 cycles for Cbfa1 (which remains in the linear range) and 15 cycles for GAPDH, followed by 72 C for 10 min. Samples were separated on 1% agarose gels. PCR products were transferred onto Hybond N^+ nylon membranes (Amersham Pharmacia Biotech) and hybridized at 65 C to a probe corresponding to the 266-bp *Bam*HI-*Nco*I-digested fragment from the N-terminal region of Cbfa1 (PEBP2αA1) cDNA. Hybridized blots were washed at 65 C with 1 × SSC and 0.1% SDS. Data were analyzed using a PhosphorImager after exposure for 2–24 h.

Immunofluorescence

Osteoblasts were grown on glass coverslips (Fisher Scientific, Springfield, NJ) at 37 C in growth medium to 40% confluence for 24 h. Cells were fixed in 4% paraformaldehyde in PBS or subjected to *in situ* extraction of cytoskeletal and soluble chromatin proteins to reveal the nuclear matrix-intermediate filament scaffold (see below). Coverslips were processed using protocols previously described by our laboratory (25).

Antibody staining was performed using an affinity-purified Cbfa1 primary antibody (38) at a dilution of 1:200 and was incubated for 1–1.5 h at 37 C. Coverslips were then incubated with a fluorescein isothiocyanate (FITC)-conjugated goat antirabbit secondary antibody (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and incubated for 1 h at 37 C to detect Cbfa1. *In situ* nuclear matrexes were prepared as previously described (43). Briefly, cells on coverslips were washed in PBS and extracted twice in cytoskeletal (CSK) buffer for 15 min each. CSK buffer contains 10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1.2 mM phenylmethylsulfonylfluoride, and 1% vanadyl ribonucleoside complex. Deoxyribonuclease I digestion was performed twice in digestion buffer (CSK buffer with 50 mM NaCl) containing 100 μg/ml deoxyribonuclease I for 30 min, followed by an extraction in digestion buffer containing 0.25 M $(NH_4)_2SO_4$ for 10 min. DNA content was evaluated by staining with 4′,6-diamidino-2-phenylindole (DAPI; 5 μg/ml in PBS containing BSA and 0.05% Triton X-100). Cells were mounted in Vectashield H-1000 (Vector Laboratories, Inc., Burlingame, CA). Images were obtained using a CCD camera interfaced with a digital microscope system (Carl Zeiss, Thornwood, NY). Images were analyzed by Metamorph software (Universal Imaging Corp., West Chester, PA).

Results

Expression of Cbfa1 isoforms during osteoblast growth and differentiation

We previously reported an increase in Cbfa1 protein and DNA binding activity by EMSA of nuclear extracts from the proliferating to differentiated stages of primary rat calvarial osteoblast culture (8). To determine whether the increased DNA binding (Fig. 1A) is regulated by modulation of protein levels, we performed Western blot analysis (Fig. 1B) with nuclear extracts isolated during growth and differentiation of primary rat osteoblasts. The Western analysis showed low Cbfa1 protein levels on d 2 and significant levels in postproliferative d 14 cells. Cbfa1 protein level was thereafter moderately increased on d 20 (Fig. 1B). These results were confirmed using different antibodies [described in Ref. 16 and obtained from Oncogene Research Products (Boston,

TABLE 1. Primers used for RT-PCR

	Genes and primer sequences	Product length (bp)	GenBank accession no.	Ref. (primers)
Cbfa1 (type II)				
Sense	5′-GAG GGC ACA AGT TCT ATC TG-3′	386	AF010284	Ducy *et al.*, 1997 (9)
Antisense	5′-GGT GGT CCG CGA TGA TCT-3′			
Cbfa1 (type I)				
Sense	5′-ATG CGT ATT CCT GTA GAT CCG AGC-3′	307	D14636	This work
Antisense	5′-GGT GGT CCG CGA TGA TCT-3′			
Rat Osteocalcin				
Sense	5′-ATG AGG ACC CTC TCT CTG CTC-3′	293	X04141	Fleet and Hock, 1994 (61)
Antisense	5′-GTG GTG CCA TAG ATG CGC TTG-3′			
Rat Alkaline phosphatase				
Sense	5′-TGG ACG GTG AAC GGG AGA A-3′	440	J03572	Noda *et al.*, 1987 (62)
Antisense	5′-GGC GGA AGT GAG GCA GGT AG-3′			
Rat GAPDH				
Sense	5′-CCA TGG AGA AGG CTG GGG-3′	194	AB017801	Fleet and Hock, 1994 (61)
Antisense	5′-CAA AGT TGT CAT GGA TGA CC-3′			
Mouse Osteocalcin				
Sense	5′-TCT GAC AAA CCT TCA TGT CC-3′	198	S67455	Qu *et al.*, 1998 (63)
Antisense	5′-AAA TAG TGA TAC CGT AGA TGC G-3′			
Mouse Alkaline Phosphatase				
Sense	5′-GCC CTC TCC AAG ACA TAT A-3′	372	J02980	Qu *et al.*, 1998 (63)
Antisense	5′-CCA TGA TCA CGT CGA TAT CC-3′			
Mouse Collagen type I				
Sense	5′-TCT CCA CTC TTC TAG TTC CT-3′	250	X06753	Qu *et al.*, 1998 (63)
Antisense	5′-TTG GGT CAT TTC CAC ATG C-3′			
Mouse Smad 1				
Sense	5′-CGA GTG GAG AGC CCG GTT CTC-3′	519	AF033116	Dick *et al.*, 1998 (64)
Antisense	5′-AAG CGG TTC TTA TTG TTG GAC-3′			
Mouse MyoD				
Sense	5′-GCA GGC TCT GCT GCG CGA CC-3′	370	M84918	Valdez *et al.*, 2000 (65)
Antisense	5′-TGC AGT CGA TCT CTC AAA GCA CC-3′			
Mouse GAPDH				
Sense	5′-CAC CAT GGA GAA GGC CGG GG-3′	418	M32599	Qu *et al.*, 1998 (63)
Antisense	5′-GAC GGA CAC ATT GGG GGT AG-3′			

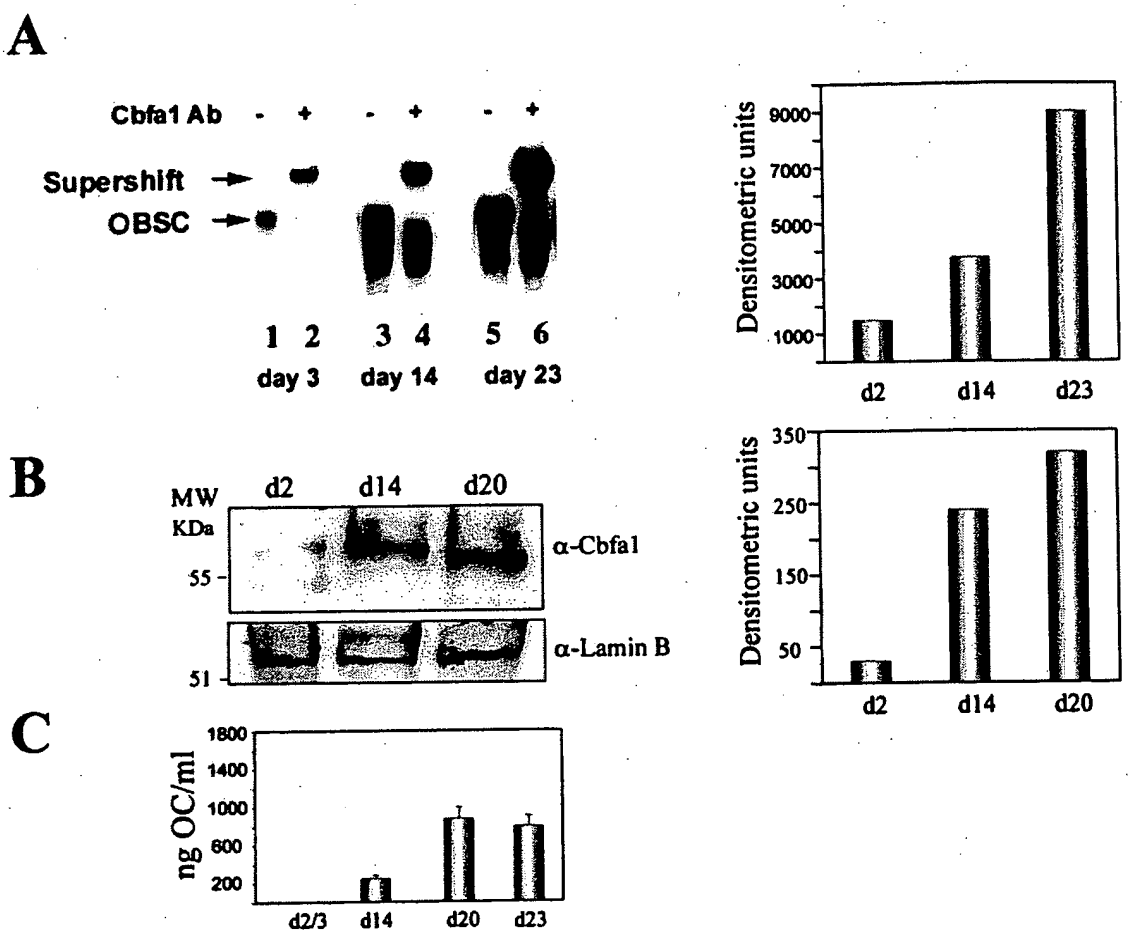


FIG. 1. Representation of Cbfa1 during the growth and differentiation of fetal rat calvarial osteoblasts. A, DNA binding activity of Cbfa1 during osteoblast differentiation. EMSAs were performed using rat osteoblast nuclear extracts (lanes 1 and 2, d 3; lanes 3 and 4, d 14; lanes 5 and 6, d 23), a probe containing the consensus Cbfa-binding site and Cbfa1 antiserum (38). Lanes 1, 3, and 5, Nuclear extracts incubated with preimmune serum; lanes 2, 4, and 6, nuclear extracts incubated with 1 μl Cbfa1 antiserum. Dried gels were exposed for 6 h. The Cbfa1-containing complex (OBSC) and the supershifted bands (supershift) are indicated. The graph to the *right* is an average of the densitometric analysis of the supershifted complex. B, Cbfa1-immunoreactive proteins during osteoblast differentiation. Western blots were performed using rat osteoblast nuclear extracts obtained from different stages of growth and differentiation (proliferating d 2, differentiating d 14, and mineralizing d 20) as indicated. The membrane was incubated with antibody as previously described (16). Similar results were obtained using antibody from Meyers *et al.* (38) and Oncogene Research Products. Relative migration of Cbfa1 and lamin B to the markers is indicated. The graph to the *right* of B presents densitometric quantitation of the Cbfa1-immunoreactive band. C, The extent of osteoblast differentiation as reflected by secreted osteocalcin measured by RIA for the experiments presented in A and B.

MA)] and with different time courses. It is noteworthy that an additional Cbfa1 minor band is present in very heavily mineralized osteoblast cultures, perhaps indicating partial degradation (data not shown). At late stages of differentiation, secreted osteocalcin levels are still in the peak range (Fig. 1C), consistent with increased Cbfa DNA binding activity (Fig. 1A). Thus, the greatest increases in Cbfa1 cellular protein occur between proliferating (d 2) and differentiated cells (d 14). However, the changes in Cbfa1 protein between d 14 and d 20 or d 23 are less dramatic than the increase observed in DNA binding activity. This discordance may reflect posttranslational modifications in the osteoblast-specific complex (OBSC) that enhance Cbfa1 DNA binding activity, which can be observed in native EMSA gels, in contrast to the denaturing conditions of the Western analysis.

To assess the cellular localization of endogenous Cbfa1 protein in proliferating osteoblasts, we performed *in situ* immunofluorescence analysis. Endogenous Cbfa1 is detected in whole cell and nuclear matrix preparations of proliferating rat osteoblasts as intense punctate staining of the protein(s) localized specifically in the nucleus (Fig. 2, A and B). Together, these results establish that all the Cbfa1 protein detected is present in the nuclei. In proliferating osteoblasts, Cbfa1 is completely localized in subnuclear domains; thus, the low protein levels and DNA binding activity are not related to partitioning between the nucleus and the cytoplasm.

To determine whether the increases in the DNA-binding complex and protein levels by Western analyses correlated with increased cellular synthesis of Cbfa1, Northern analysis was performed on RNA derived from time courses of primary rat osteoblasts (ROB) and mouse MC3T3-E1 preosteoblasts (Fig. 3). We hybridized blots of ROB total RNA (from d 2, 7, 11, 18, and 22; Fig. 3, A–C) with a full-length cDNA

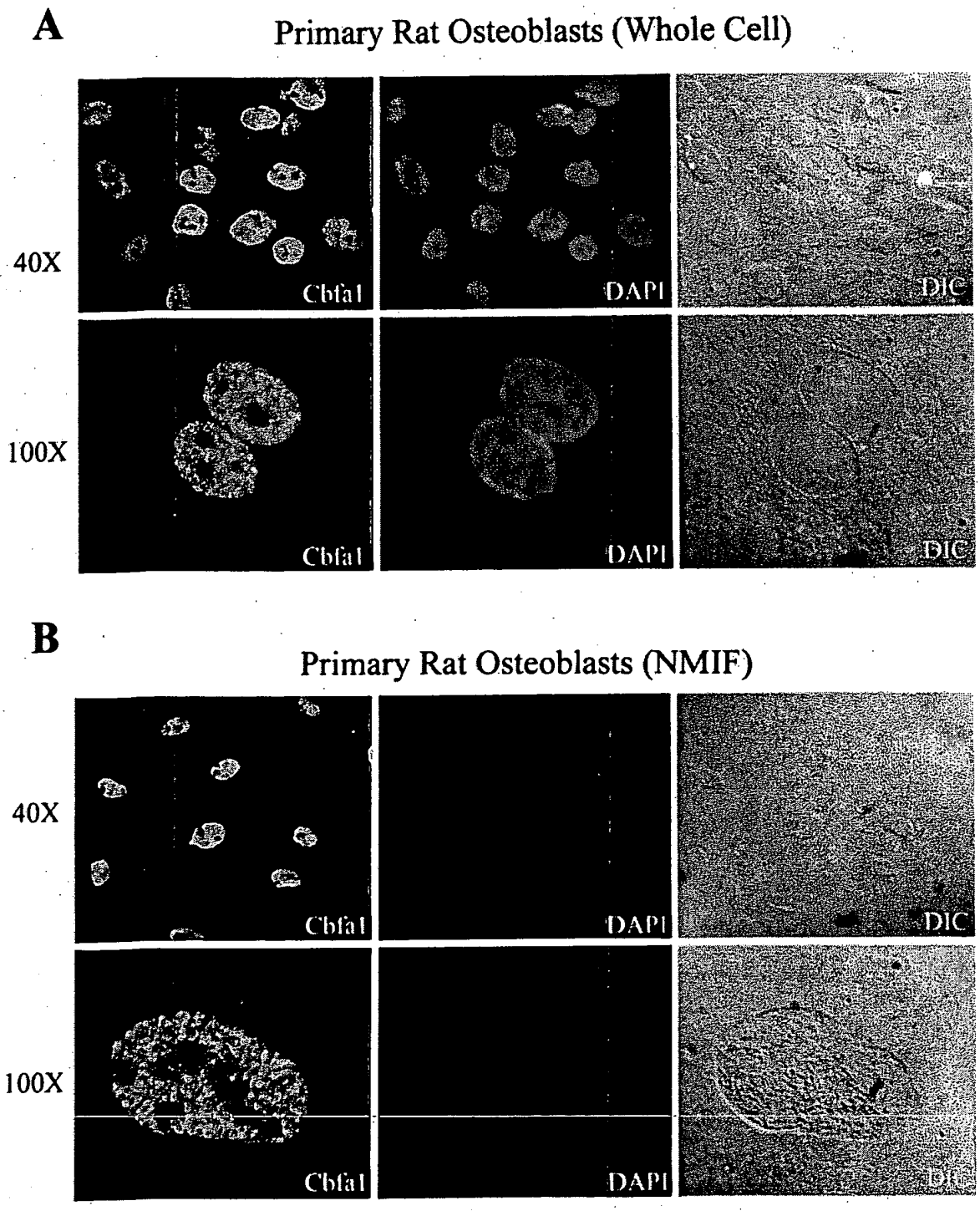


FIG. 2. Immunofluorescence detection of endogenous Cbfa1 in rat calvarial osteoblasts. Whole cell and nuclear matrix preparations of primary osteoblasts (d 2) were fixed in 4% paraformaldehyde, washed, and incubated with anti-Cbfa1 primary antibody followed by FITC-conjugated goat antirabbit secondary antibody as described in *Materials and Methods*. Representation of Cbfa1 in whole cells (A) and *in situ* nuclear matrix (NMIF) localization (B) at 40× (*top rows*) and 100× (*bottom rows*) are shown. *Left panels*, FITC-stained Cbfa1; *middle panels*, DAPI-stained nuclei; *right panels*, differential interference contrast (DIC) image of the cells. The absence of DNA in DAPI in B indicates the complete removal of chromatin from nuclear matrix intermediate filament (NMIF) preparation.

probe with the potential to recognize all the Cbfa1 mRNAs (Fig. 3A), as well as with an exon 1 probe (Fig. 3B), which should specifically detect mRNAs encoding type II isoforms (illustrated in Fig. 4A). Our results show that multiple mRNA transcripts ranging in size from 0.6–6 kb are detected with the full-length probe (Fig. 3A), and there is a developmental increase in the major transcript from growth to differentiation stages of rat osteoblasts. Using the exon 1-specific probe,

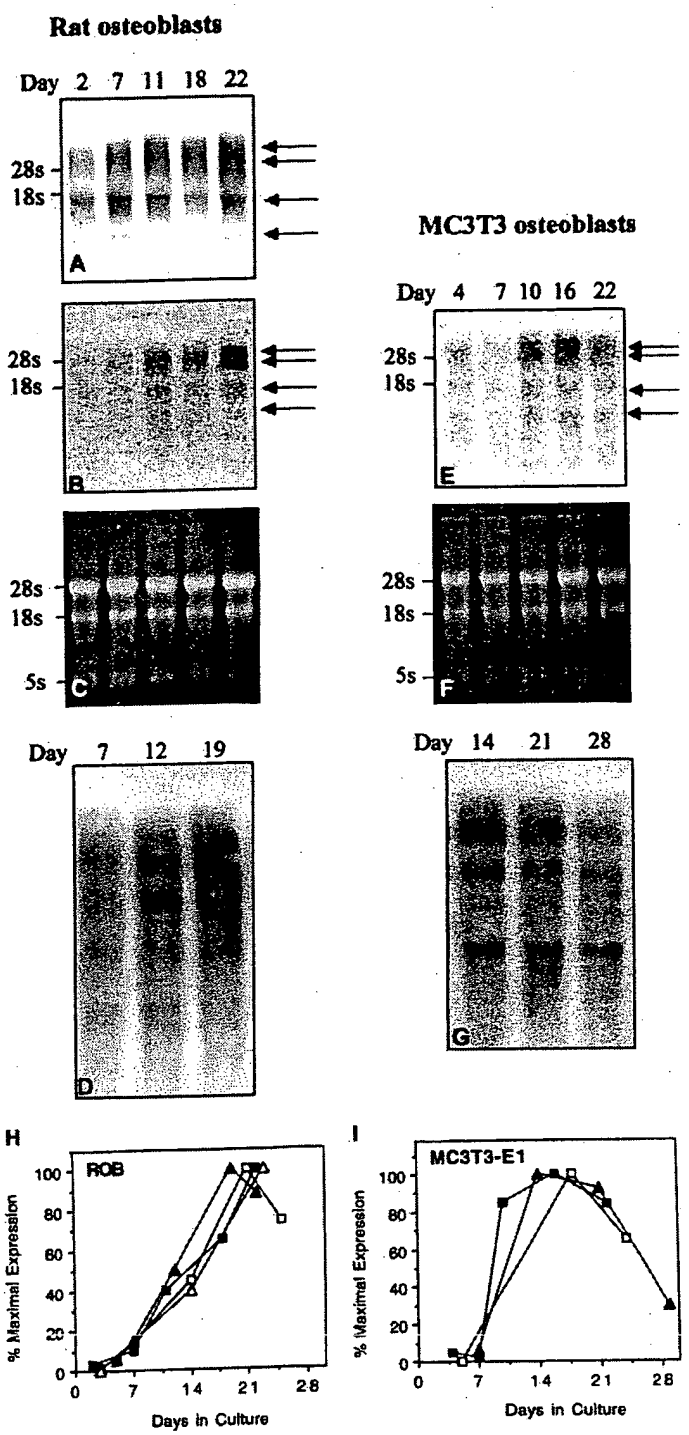


FIG. 3. Northern analysis of Cbfa1 transcripts during osteoblast differentiation. Total cellular RNA was isolated during different stages of growth and differentiation from primary ROB (d 2, 7, 11, 18, and 22; A–C) and mouse MC3T3-E1: d 4, 7, 10, 16, and 22 (E and F). RNA (10 μg) was electrophoretically resolved in a 1% agarose/formaldehyde gel and blotted onto a nylon membrane. Hybridizations were performed with a full-length Cbfa1 cDNA probe (A) or a 460-bp probe corresponding to the *Eco*RV-*Bam*HI fragment containing the exon 1 sequence of Cbfa1 cDNA (B and E). The blot was analyzed in a PhosphorImager after 18-h exposure. Cbfa1 transcripts indicated by *arrows*; 28S and 18S rRNA markers are indicated. Ethidium bromide staining of the same gels is shown in C and F to demonstrate the amount of RNA loading in each sample. D (ROB cells) and G (MC3T3E1 cells), Northern blot analyses of poly(A)$^+$ RNA from a different time course (the indicated days are shown) to verify the multiple transcripts and developmental changes during osteoblast differentiation observed with total cellular RNA. The full-length Cbfa1 probe was used for hybridization. H (ROB) and I (MC3T3E1) summarize the combined expression of the major Cbfa1 transcripts during osteoblast differentiation [*uppermost two arrows* in B (rat) and D (mouse) in three different time courses]. The values from independent time courses (n = 4, ROB; n = 3, MC3T3) are plotted as the percent maximal expression in each time course.

the increase in the major transcript above the 28S marker becomes more evident (Fig. 3B). Northern analysis of poly A$^+$ RNA using full-length Cbfa1 cDNA probe (Fig. 3D) confirmed the presence of multiple transcripts and their increased expression during osteoblast differentiation.

We also examined the developmental profile of Cbfa1 RNA expression during the differentiation of mouse osteoblasts on d 4, 7, 10, 16, and 22 (Fig. 3, E–G). The MC3T3-E1 cell line has been well characterized with respect to the stages of osteoblast maturation (44). Using the same exon 1-specific probe, we observed that type II Cbfa1 RNA levels increased from proliferating (d 4–7) to postproliferative (d 10) and mineralizing (d 16–22) MC3T3-E1 cultures. However, consistent with other time courses, we observed a modest decline after d 22 when the matrix was heavily mineralized in the mouse cell cultures. Other mRNAs (*e.g.* osteocalcin) also declined. Northern blot analysis of poly(A)$^+$ RNA with a full-length cDNA probe (Fig. 3G) confirmed our findings that multiple Cbfa1 transcripts are expressed, similar to total cellular RNA in mouse osteoblasts (Fig. 3E). Thus, the cellular levels of Cbfa1 transcripts that are derived from exon 1 increase in expression and are dramatically up-regulated during the onset of differentiation in both calvarial rat and mouse osteoblasts (Fig. 3, H and I, summary of data from several time courses).

To further confirm the specific development patterns of expression of the Cbfa type I and type II transcripts during osteoblast differentiation, we performed RT-PCR analysis with primers (shown in Table 1) specific for exon 1 or exon 2 (Fig. 4). Semiquantitative RT-PCR of mRNA from primary rat osteoblasts (Fig. 4B) and mouse MC3T3 cells (Fig. 4C) indicated that transcripts of both Cbfa1 isoforms were detected throughout the courses of osteoblast differentiation. Although the mRNA encoding type I was constitutively expressed from growth to mineralization stages, the type II encoded transcript increased postproliferatively from d 2 to 14/d 23 in rat osteoblasts (Fig. 4, B and C) and from d 7 to d 10/d 16 in MC3T3E1 cells (Fig. 4D). Notably, we observed a decline in representation of the type II isoform in heavily mineralized mouse osteoblasts (d 22; Fig. 4, D and E), consistent with Northern blot analyses (Fig. 3, E and G). We confirmed the specificity of the rat osteoblast RT-PCR products (visualized in agarose gels) by Southern hybridization with a Cbfa1-specific cDNA probe (described in *Materials and Methods*; Fig. 4B, *right panel*). For comparison, osteoblast phenotypic markers alkaline phosphatase (ALP) and OC are shown to reflect osteoblast differentiation, and as expected, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is constitutive. Taken together, these results show constitutive expression of type I and develop-

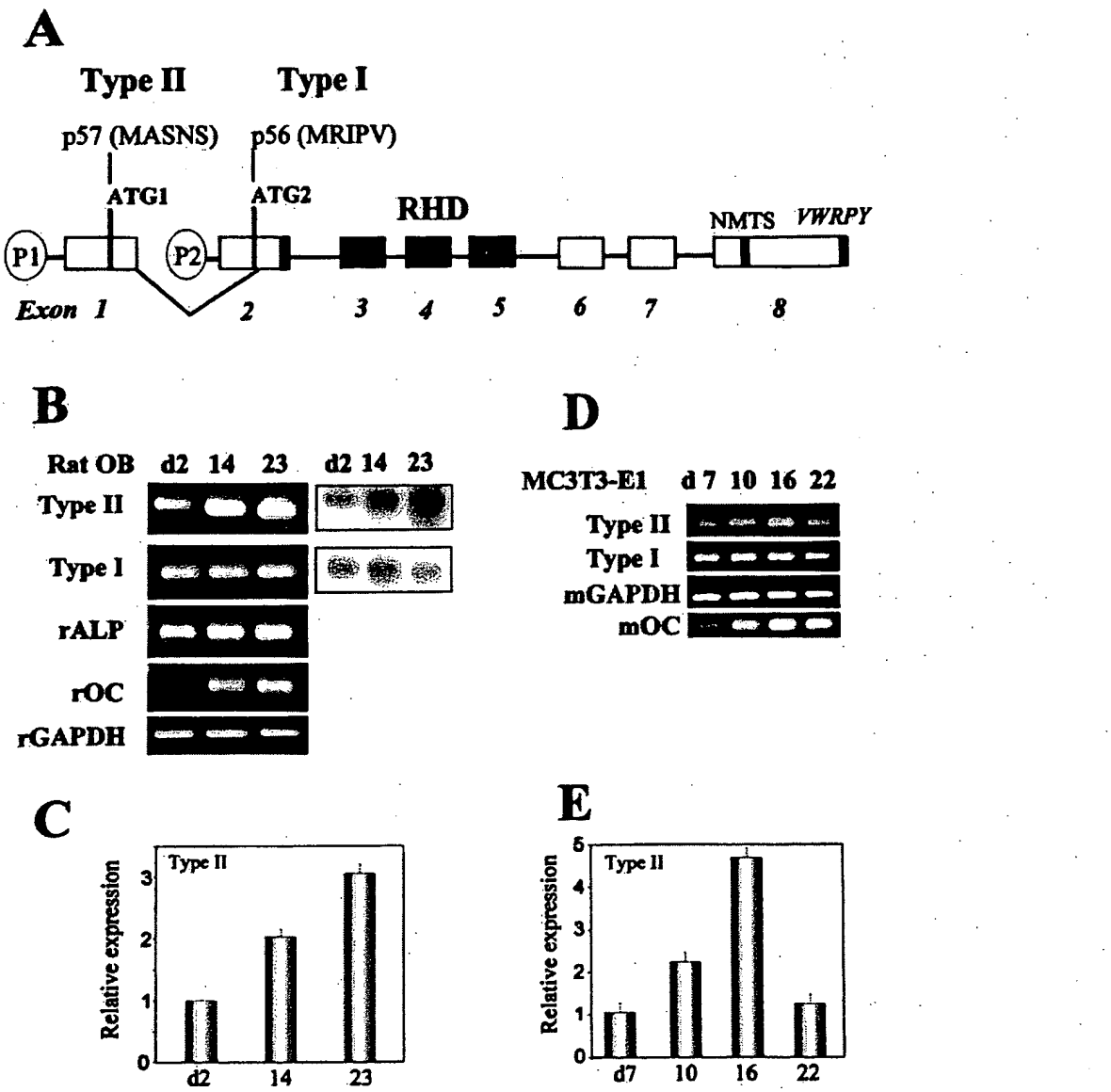


FIG. 4. Expression of Cbfa1 transcripts during growth and differentiation of rat osteoblasts. A, Runx2/Cbfa1 gene organization, indicating the derivation of the principal N-terminal isoforms. Schematic illustration of exons 1–8 that comprise the Cbfa1 gene and the exon origin of the Cbfa1 isoforms p57 (MASNS), type II and p56 (MRIPV), or type I. Exon 1 shows the ATG1 start site for the type II isoform (*til-1*) (14), starting with the sequence MASNS, and exon 2 contains the start site of type I isoform (PEBP2aA1) (17), starting with the sequence MRIPV. The *solid black* exons denote the runt homology DNA binding domain (RHD); NMTS designates the location of the 31-amino acid nuclear matrix-targeting signal (43). The conserved VWRPY motif at the extreme C-terminal region of Cbfa1 gene is indicated. B, Total RNA from primary rat osteoblasts (proliferating d 2, postproliferative d 14, and differentiated in a mineralizing matrix d 23) were reverse transcribed followed by PCR amplification using Cbfa1 type II and type I isoform-specific primers and separated in 1% agarose gels as described in *Materials and Methods*. Rat ALP and rat OC expression are shown for reference. The rat GAPDH transcript indicates equal loading of samples. Cbfa1 type II and type I RT-PCR products were further confirmed by Southern hybridization: shown in panels on the *right* (see *Materials and Methods*). C, Graphic representation of the developmental expression of the type II Cbfa1 isoform in ROB cells. Data from two different time courses are plotted as the percent maximal expression from quantitative densitometric analysis of Southern blots of the RT-PCR products. Values are the mean ± SD. D, RT-PCR performed on MC3T3 cell-derived total RNA from proliferating through differentiated stages (d 7, 10, 16, and 22) as indicated. PCR amplification was performed using type II and type I isoform-specific primers and separated on a 1% agarose gel as described in *Materials and Methods*. Mouse GAPDH serves as a control for equal loading, and OC was used as reference for differentiation. E, Graphic representation of the developmental expression of the type II Cbfa1 isoform in differentiating MC3T3-E1 cells. Calculations are the same as those described in C.

mental and preferential expression of type II in final stages of osteoblast differentiation Thus, it appears that the two isoforms together account for Cbfa1 protein levels by EMSA, Western, and immunofluorescence analyses.

To ascertain the tissue specificity of Cbfa1 type I and II isoforms, we examined their expression in nonosseous organs and primary cultures of cells from soft tissues of the newborn mouse. We examined total cellular RNA from four organs, liver, lung, muscle, and skin, and the primary cells cultured to confluence (Fig. 5). The type I isoform, but not the type II isoform, was present in primary cell cultures as well as in RNA from the tissues. The RT-PCR product for the type

I isoform was consistently detected at very low levels in liver tissue, but at significant levels in the mesenchymal tissues, particularly skin. Thus, the Cbfa1 type I isoform, which was shown to be present in thymus and spleen (1, 20), appears to be more ubiquitously expressed than previously appreciated.

Functional analyses of type I and type II isoforms

The type I and type II isoforms each have distinct N-termini (Fig. 4) regulated by distinct promoters and the N-terminus of the type II isoform is 15 amino acids longer. However, the functional consequences of these molecular differences have not been unequivocally established. To assess the transcriptional activities of the type II and type I isoforms, we performed transient cotransfection studies with these two isoforms and a panel of bone-related Cbfa-responsive target genes (*e.g.* OC, TGFβ-RI, and bone sialoprotein). Transfections were performed with osteoblastic ROS 17/2.8 cells (Fig. 6B) and HeLa cells that do not contain Cbfa1 (Fig. 6A). In both cell types we observed that type I and type II isoforms activated the OC and TGF-βRI promoters by 4- to 8-fold and repressed bone sialoprotein promoter activity by 4- to 8-fold. No statistically significant differences were observed in the activation or repression potential of the two isoforms, and both transfected proteins were expressed at similar levels (Fig. 6C). The similarities in transcriptional results for both isoforms suggest that the 15-amino acid extension of the type II isoform does not contribute to activation or repression of transcription. Our results demonstrate that the type I and type II isoforms have nearly equivalent transcriptional activities.

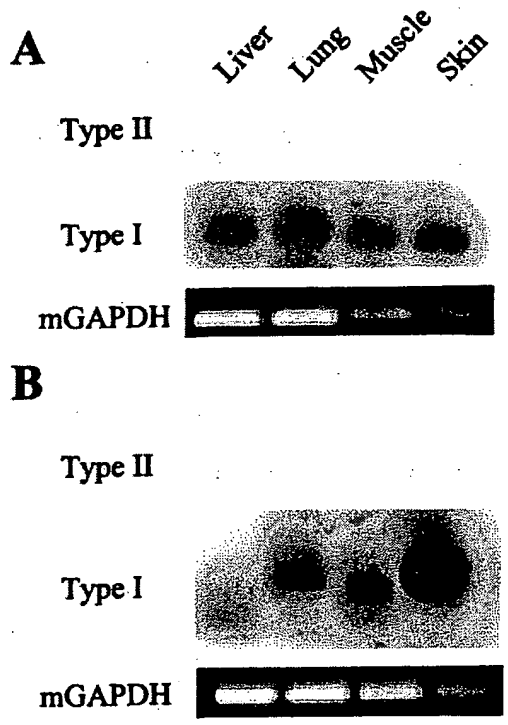


FIG. 5. Cbfa1 type I isoform, but not type II isoform, is present in mesenchymal tissues. RT-PCR for Cbfa1 isoforms in primary cultures of cells from the tissues indicated (A) and mouse tissues (B). The primers used are shown in Table 1, and the conditions for RT-PCR are described in *Materials and Methods*. GAPDH is shown for control levels.

Cbfa1 type I and type II isoforms are both expressed in skeletal progenitor cells and are differentially regulated by BMP-2

The presence of significant levels of Cbfa1 protein in proliferating osteoblasts, which are precommitted cells derived from calvarial bone, led us to assess whether the Cbfa1 gene is expressed at earlier stages of osteoblast development. We examined cellular Cbfa1 protein levels in the skeletal progenitor cells MLB13 MYC clone 14 and MLB13MYC clone 17 (32) derived from mouse limb bud. MLB13MYC clone 14 represents an undifferentiated early skeletal progenitor that differentiates into chondroblasts and then into osteoblasts in response to BMP-2. MLB13MYC clone 17 represents a prechondroblast cell line that directly differentiates into osteoblasts after BMP-2 treatment. Cbfa1 proteins were initially analyzed by whole cell *in situ* immunofluorescence microscopy. In both cell lines, endogenous Cbfa1 proteins were detected throughout the cell layer and exhibited intense punctate FITC staining in the nucleus (Fig. 7, A and B). Thus, Cbfa1 proteins are uniformly and highly expressed in these endochondral skeletal cells, which are not yet committed to the osteoblast lineage.

The presence of Cbfa1 proteins in early mesenchymal progenitor cells suggests that it may have an important role in early stages of lineage commitment, but our data do not discriminate whether one or both Cbfa1 isoforms are expressed. Therefore, we examined representation of Cbfa1 type I and type II transcripts in these two cell lines after BMP-2-induced osteoblast differentiation. Semiquantitative RT-PCR analyses (Fig. 8A) showed that both type I and type II transcripts were detected at low levels in the clone 14 and clone 17 cells before BMP-2 treatment, when cell phenotype-specific markers are not expressed (*e.g.* mALP and mOC). BMP-2 treatment for 48 h significantly enhanced the expression of the type II transcript, but not the type I transcript, in both cell lines. In parallel, expression of bone phenotype transcripts mOC and mALP increased with BMP-2 treatment, indicating differentiation of the skeletal progenitor cells, whereas expression of the housekeeping gene mouse GAPDH remained constant. Hence, our data clearly show selective up-regulation of the type II isoform along with onset of expression of bone phenotypic markers.

To evaluate whether up-regulation of type II expression after BMP-2 treatment increased Cbfa1 DNA binding activity, we analyzed nuclear extracts from these two skeletal progenitor cells for formation of DNA binding complexes with the Cbfa1 consensus binding site as the probe. EMSAs coupled with Cbfa1 antibody supershift assays demonstrate that both of these cell lines form Cbfa1-containing DNA binding complexes (Fig. 8B). Treatment of cells with BMP-2 substantially increased the Cbfa1 protein-DNA complex. This result parallels the observed increase in the type II transcript in differentiated rat and mouse osteoblasts detected by RT-PCR (Fig. 4). Thus, these biochemical studies reveal that Cbfa1 proteins are expressed in very early stage mesenchymal progenitors, before their differentiation into

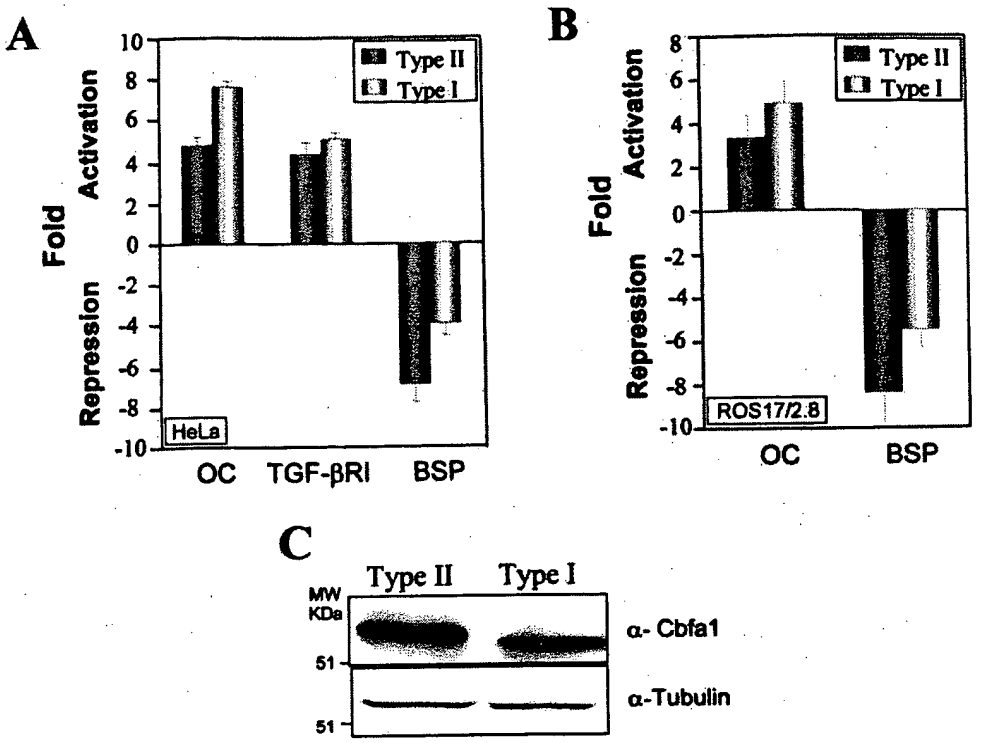


FIG. 6. Activity of Cbfa1 isoforms on promoters of genes expressed in osteoblasts. A, Transfections were performed in HeLa cells that do not endogenously express Cbfa factors. The cells were plated in six-well plates and transiently cotransfected with expression vector for type I or type II isoforms (750 ng/well) together with either 2.5 μg/well 1.1 kb OC promoter (OC), 1.6 kb TGF-βRI, or 0.6 kb bone sialoprotein promoter (BSP) fused to the *luciferase* reporter gene. The fold activation for OC and TGF-βRI and the fold repression for BSP are shown for n = 6. B, Rat osteosarcoma ROS 17/2.8 cells were transiently transfected as described for HeLa cells. Fold activation or repression for six values are shown. C, Western analyses shows Cbfa1 protein expression in HeLa cell lysates (30 μg protein) harvested 24 h after transfection with either the type II or type I Cbfa1 isoforms and compared with tubulin as a control.

the osteoblast phenotype. The enhanced expression of the type II transcript in response to BMP-2 correlates with increased DNA binding activity that parallels induction of osteoblast differentiation by BMP-2.

BMP-2 induces selective expression of the type II Cbfa1 isoform during osteogenic differentiation of myogenic progenitor C2C12 cells

The presence of Cbfa1 type II isoform in endochondral progenitor cells suggests that expression of the type II isoform precedes cellular commitment to osteoblasts and/or is a marker for cells destined to differentiate into bone cells. To address this question, we examined the C2C12 cell model, a premyoblastic cell line of mesenchymal origin, because muscle cells lack expression of Cbfa1 type II isoform (Fig. 5), and the C2C12 line is capable of *trans*-differentiating into the osteoblast lineage in response to BMP-2 (33). We used RT-PCR to assess expression of the Cbfa1 type I and type II isoforms in the myoblast stage and during BMP-2-induced osteoblast differentiation (Fig. 9). These studies were carried out in 5% serum, which supports myogenesis, and 10% serum, which favors osteogenic differentiation. In parallel, cells were also analyzed in the presence of charcoal treated FBS (stripped serum). Figure 9 demonstrates that expression of the type II isoform was not detected in control C2C12 cells cultured in either 5% or 10% serum (regular or stripped). Interestingly, after 48 h of treatment with BMP-2, we observed a significant increase in expression of the type II transcript in cultures grown in either 5% or 10% serum, although the increase was greater in 10% serum. Notably, the type I transcript was expressed in the C2C12 cell line before treatment with BMP-2 (see control lanes). Expression of type I did not significantly differ between control and BMP-2-treated cells in either 10% or 5% serum in repeated studies; however, in stripped serum, BMP-2 stimulated the type I RT-PCR product. This finding indicates that factors present in regular serum may influence the expression of this isoform. As expected, the expression of myogenic MyoD was suppressed by BMP-2 in cultures supplemented with either 5% or 10% serum. This down-regulation of MyoD with BMP-2 treatment occurred concomitant with induction of the osteoblast marker OC, whereas Smad 1, a downstream target of the BMP-2 signaling pathway, showed no appreciable change in expression, as expected (45). Together, these results demonstrate that the expression of Cbfa1 type II isoform is induced with onset of the osteoblast phenotype (that is, expressing OC), whereas the type I isoform is expressed in mesenchymal progenitors and is not restricted to skeletal tissues.

Discussion

Although genetic studies have shown that Cbfa1 is essential for bone formation (3–5), these approaches did not distinguish the biological roles of the two different N-terminal isoforms of Cbfa1 in transcriptional control of osteoblast differentiation. In this study we have characterized the expression pattern of each of the major N-terminal isoforms in models of osteogenic differentiation. Our findings can be summarized as follows. First, the type I isoform is expressed before the type II; second, type I transcript expression is ubiquitous and constitutive in both nonosseous and mesenchymal cells and during all stages of osteoblast differentiation; and third, a developmental increase in type II transcript expression occurs during differentiation of primary osteoblasts and after BMP-2 mediated differentiation of progenitor cells. We propose that the Cbfa1 type I isoform represents

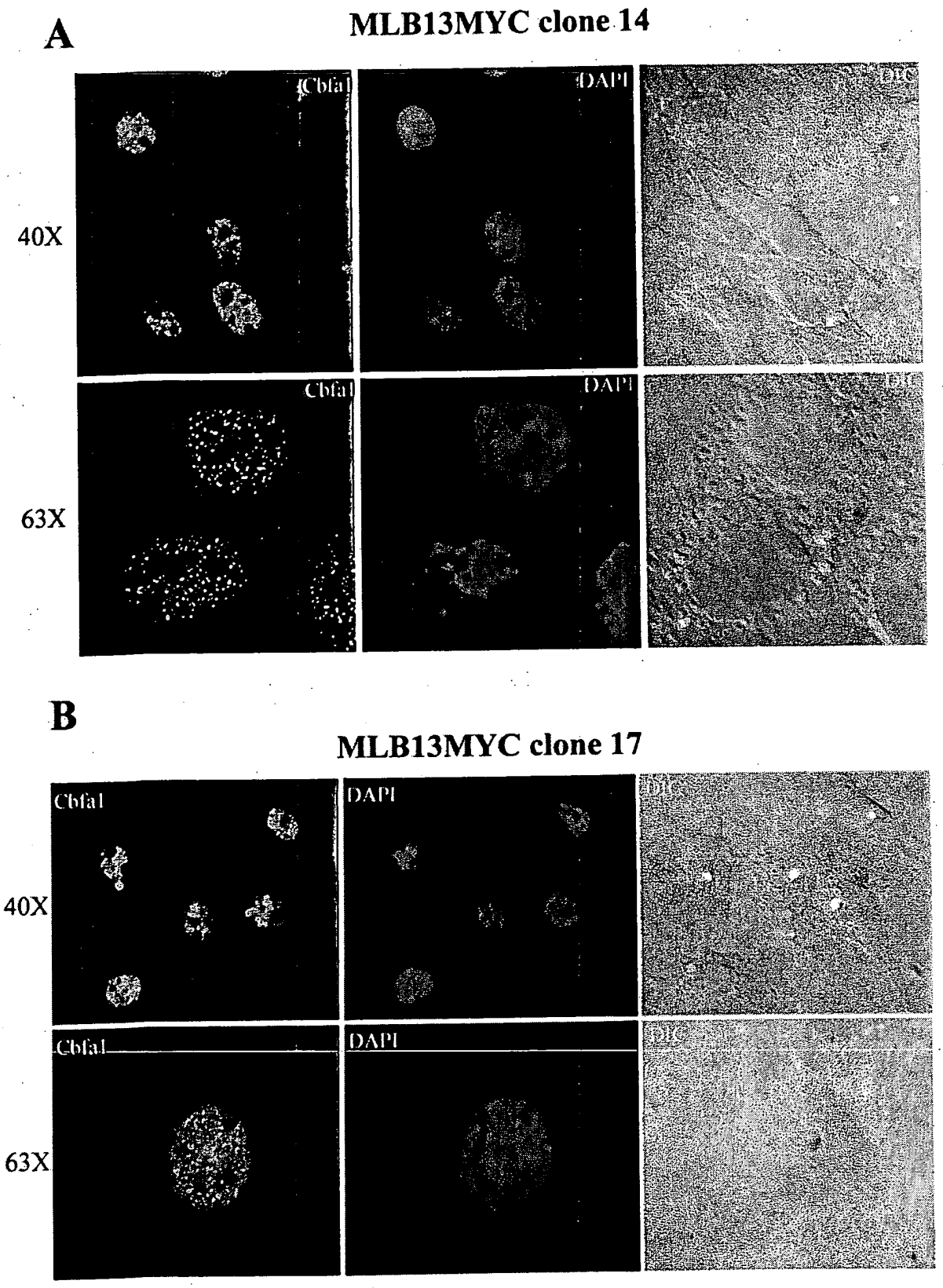


FIG. 7. Cellular levels of Cbfa1 proteins in mouse marrow-derived progenitor cell lines. Cells were fixed with 4% paraformaldehyde, washed, and incubated with Cbfa1 primary antibody followed by FITC-conjugated goat antirabbit secondary antibody as described in *Materials and Methods*. Endogenous expression of Cbfa1 in whole cells of MLB13MYC clone 14 (A) and MLB13MYC clone 17 (B) at 40× and 63× magnifications are shown as indicated. Cbfa1 expression is shown in the *left panels*; DAPI staining of nucleus (*middle panels*) and differential interference contrast (DIC) (*right panels*) images for both fields are shown.

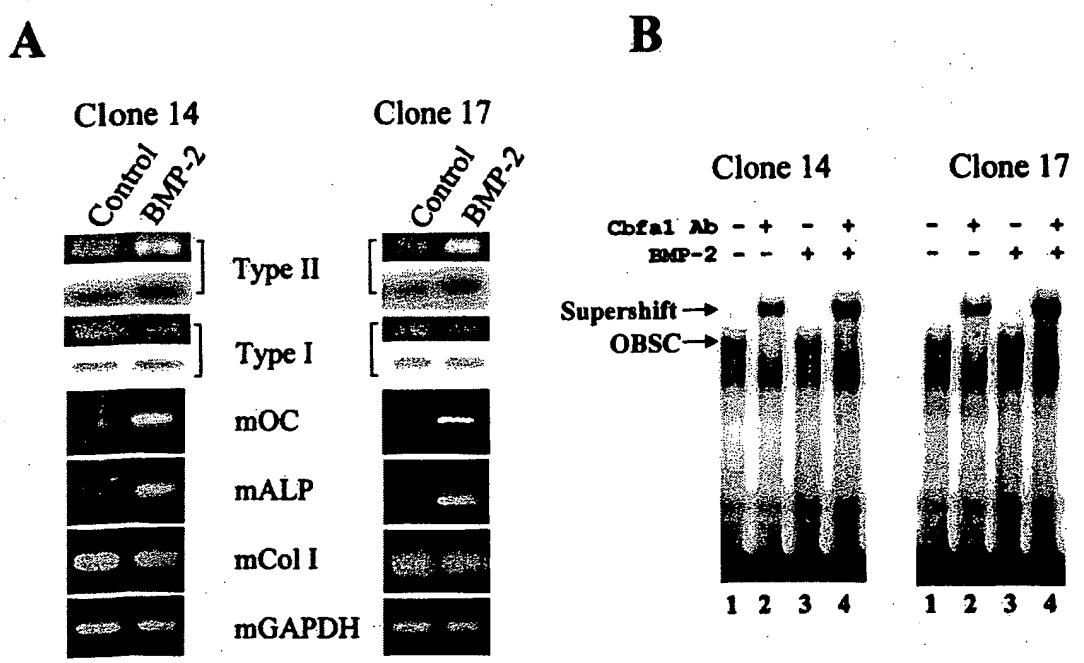


FIG. 8. Detection and BMP-2 regulation of Cbfa1 isoforms in early mouse progenitor cells. A, RT-PCR analysis of Cbfa1 transcripts. MLB13MYC clone 14 and MLB13MYC clone 17 cells with or without 48-h BMP-2 treatment (100 ng/ml) were harvested on d 7. Total RNA isolated from untreated (Control) and treated (BMP-2) cells were reverse transcribed, followed by PCR amplification using type I and type II Cbfa1isoform-specific primers (Table 1) as described in *Materials and Methods*. For type I and type II Cbfa1, PCR products were hybridized with Cbfa1 cDNA (*lower panels*) to indicate the specificity of the reaction products. Mouse OC (mOC), mALP, and mouse collagen I (mCollI) are used as reference for osteoblast differentiation and equal sample loading represented by mGAPDH. B, BMP-2 increases the DNA binding activity of Cbfa1 in skeletal progenitor cells. EMSA nuclear extracts obtained from skeletal progenitor cell lines MLB13MYC clone 14 and MLB13MYC clone 17 were incubated with anti-Cbfa1 antiserum in binding reactions using a probe containing the consensus Cbfa binding site. Lanes 1 and 2, Nuclear extracts from untreated control cells; lanes 3 and 4, nuclear extracts from cells treated 48 h with BMP-2 (100 ng/ml). Reactions in lanes 2 and 4 were incubated with Cbfa1 antiserum (38). Dried gels were exposed for 12–16 h. The OBSC and the Cbfa1 supershifted bands (supershift) are indicated.

a marker of early stage stromal mesenchymal cells, and that type II isoform defines a cell that has the potential for commitment to the osteoblast lineage. Our data also suggest that Cbfa1 contributes to gene regulation for osteoblast differentiation through multiple levels of control.

The intricate regulatory pattern of Cbfa1 isoform expression is generated at least in part by utilization of alternative ATG start codons located in different exons and transcribed from two distinct promoters (11, 14, 19). We detected at least two major long transcripts and several minor shorter transcripts by Northern analysis using the full-length Cbfa1 cDNA as probe. Different mRNA variants, which represent utilization of the two promoters as well as alternative splicing and exon skipping (11, 14, 19), have been documented for other Cbfa factors (12, 46). Cbfa2, a key determinant for hemopoiesis (47), is also encoded by multiple transcripts produced through alternate promoter usage and exon skipping (46). Interestingly, alternatively spliced smaller transcripts of the Cbfa1 type II form are predominant in the testis (48). Differential regulation by two alternative promoters has also been observed for other genes. The human c-*src* gene (49) and the rat bone/liver/kidney/placenta *ALP* gene (50) each contain two alternative promoters and associated exons that splice to a common downstream exon, resulting in identical coding regions with different 5′-ends. Also, tissue-specific expression of the c-*myc* gene in *Xenopus* is controlled by two differentially regulated promoters (51). The expression of Cbfa1 variants from distinct promoters represents a versatile mechanism for temporal and cell-type specific control of Cbfa1 activity during development and for responsiveness to osteogenic factors during bone formation.

The results from our studies as well as those of others have indicated that there is considerable complexity in the expression, molecular characteristics, and biological roles of Cbfa proteins (1, 2, 11, 18, 24). Our studies show that Cbfa-dependent gene regulation involves transcriptional control of Cbfa1, cellular levels of the protein, and posttranslational formation of multiple Cbfa1-containing protein-DNA complexes. Northern blot and RT-PCR analyses of total cellular RNA from primary rat osteoblasts and mouse MC3T3E1 cells revealed increased type II RNA during progressive development of the osteoblast phenotype. The developmental increase in type II mRNA is paralleled by a similar increase in Cbfa1 protein and DNA binding activity of the osteoblast-specific Cbfa1-containing complex during early stages of mature osteoblast differentiation. However, there is a decline in type II expression in heavily mineralized stages of mouse MC3T3 osteoblasts. This decrease may be due to autoregulation of Cbfa1 (13) or may result from apoptosis of cells *in vitro*. A decline in Cbfa1 mRNA levels has been observed during cellular aging of human trabecular osteoblasts (52).

Although it is not possible to correlate specific transcripts with proteins identified by antibodies (by Western or gel shift analyses), our data indicate that transcription of Cbfa1 is rate

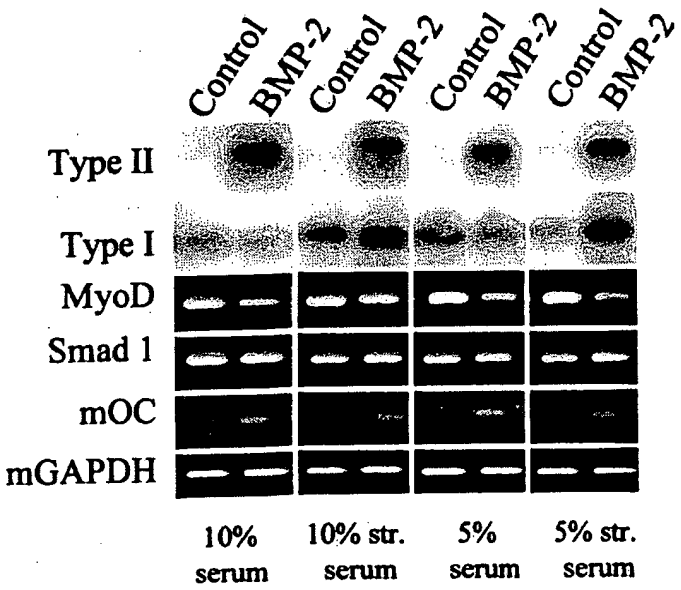


FIG. 9. Detection of Cbfa1 transcripts and protein in mesenchymal C2C12 cells. C2C12 cells were grown for 3 d in 5% or 10% serum (regular or stripped serum) and then supplemented with 300 ng/ml BMP-2 for 48 h. Total RNA from these cultures were used to perform semiquantitative RT-PCR using type I and type II Cbfa1 primers (Table 1) as described in *Materials and Methods*. Blots were hybridized to Cbfa1-specific cDNA probe to indicate the specificity of the reaction products. MyoD, Smad 1, mouse OC (phenotypic markers), as well as mouse GAPDH (for quantitation purposes) were reverse transcribed, followed by PCR using the primers listed in Table 1.

limiting for cellular protein and DNA binding activity from the immature osteoblast stage (proliferating) to postproliferative differentiated cells. However, we demonstrate a discordance between expression and protein levels relative to DNA binding in mature osteoblasts only after d 14/d 16. We suggest that Cbfa1 cellular protein is rate limiting for formation of the DNA binding complex in early stage osteoblasts (d 2–14), whereas in late stage osteoblasts, protein-protein interactions or the known posttranslational modifications of Cbfa1 may contribute to enhanced DNA binding activity. Indeed, studies have shown that phosphorylation by MAPK pathways plays a role in regulation of Cbfa1 transcriptional activity (53). Alternatively, the more significant increase in DNA binding occurring in mature osteoblasts may involve Cbfa1 interactions with numerous coregulatory proteins (2, 25). Posttranscriptional regulation or protein-protein interactions with cell type-specific cofactors may also play a major role in tissue-specific expression and activity of the Cbfa1 isoforms. Thus, the significant increase in Cbfa1 DNA binding activity in mature osteoblasts indicates that Cbfa1-interacting proteins may contribute to the regulation of a broad spectrum of genes in osteoblasts and other cell types (25, 26).

Previous *in vivo* studies have documented that during embryonic development and fracture repair, Cbfa1 is expressed in mesenchymal cell condensations of the early developing mouse (embryonic day 10) skeleton (3–7, 9, 10, 54). Cbfa1 is present in abundant levels in thymus and T and B cells (17, 55) and is also detected in stromal populations derived from human and mouse bone marrow (56), representing both fully differentiated and intermediate preosteoblastic cells (57), as well as in nonosteogenic clonal colonies (58). The significance of our findings relative to those of previous studies is that we have clearly distinguished the expression patterns of Cbfa1 type I and type II transcripts during commitment and development of the osteoblast phenotype. We demonstrate the presence of the Cbfa1 type I isoform in nonosseous cells of mesenchymal origin and constitutive expression in uninduced skeletal progenitors and early stage proliferating osteoblasts that do not yet express genes reflecting mature osteoblasts (*e.g.* bone sialoprotein, ALP, and OC) (31, 32, 44). Thus, our studies show that the type I isoform is expressed ubiquitously among cells of mesenchymal origin. We suggest that the Cbfa1 type I isoform appears in early stages of fetal development to provide pluripotent stem cells with the option for commitment to the mesenchymal lineage.

We have provided novel evidence to support the concept that p57/type II isoform of Cbfa1 is specifically related to osteogenic commitment and differentiation, because the type II transcript is selectively up-regulated by BMP-2 and during development of the osteoblast phenotype. Interestingly, we have examined 3 kb of the Cbfa1 type II promoter (13) for BMP-2 responsiveness, and under the conditions of these experiments in which endogenous mRNA and protein are increased, BMP-2 did not mediate a change in the activity of this segment of the promoter (unpublished observations). Our finding that the Cbfa1 type II isoform is selectively induced during BMP-2-mediated osteogenesis in skeletal progenitor cells and nonosseous mesenchymal C2C12 cells clarifies the differences reported in other studies. Gori *et al.* (29) showed up-regulation of Cbfa1 in response to BMP-2 in immortalized marrow stromal osteoprogenitor cells that already expressed osteoblast phenotypic markers (59). Another study showed that Cbfa1 mRNA is unaffected by BMP-2 treatment in both skeletal and nonskeletal cells, whereas BMP-4/-7 treatment up-regulates Cbfa1 expression in the same cells (21). Thus, future studies must consider interpretation of results within the context of a particular BMP, the cell type, and the specific Cbfa1 isoform.

In summary, our studies demonstrate expression of the p56/type I isoform of Cbfa1 in a spectrum of nonosseous, pluripotent, and committed osteoprogenitor cells before expression of the p57 type II isoform. We observe the regulated expression of the p57/type II isoform in committed osteoprogenitor cells and in osteoblasts as well as after BMP-2-mediated osteoblastic induction of stromal cells or *trans*-differentiation of C2C12 cells. The data indicate that BMP2-mediated induction of the Cbfa1 type II isoform through utilization of promoter 1 is critical for differentiation to osteoblasts. Our comparative expression studies indicate that although the two N-terminal isoforms are differentially expressed, they have functionally equivalent transcriptional activity on promoters in both osseous and nonosseous cell lines. Recent studies *in vivo* support this concept (60). Thus, the timing of expression of each Cbfa1 isoform, perhaps in conjunction with responses to BMPs in different subpopulations of cells during specific stages of bone development, must be an important component of the mechanism by which Cbfa1 regulates osteogenesis.

Acknowledgments

Received October 9, 2000. Accepted May 10, 2001.

Address all correspondence and requests for reprints to: Dr. Jane B. Lian, Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655. E-mail: jane.lian@umassmed.edu.

This work was supported in part by NIH Grants AR-39588, DE-12528, and AR-45688. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. The nomenclature committee of the Human Genome Organization has recently adopted the following designations for Runt-related transcription factors: RUNX1 (AML1/CBFA2/PEBP2αB), RUNX2 (AML3/CBFA1/PEBP2αA), and RUNX3 (AML2/CBFA3/PEBP2αC).

References

1. **Speck NA, Stacy T** 1995 A new transcription factor family associated with human leukemias. Crit Rev Eukary Gene Expr 5:337–364
2. **Westendorf JJ, Hiebert SW** 1999 Mammalian runt-domain proteins and their roles in hematopoiesis, osteogenesis, and leukemia. J Cell Biochem 32–33(Suppl):51–58
3. **Komori T, Yagi H, Nomura S, et al.** 1997 Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764
4. **Mundlos S, Otto F, Mundlos C, et al.** 1997 Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 89:773–779
5. **Otto F, Thornell AP, Crompton T, et al.** 1997 *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771
6. **Inada M, Yasui T, Nomura S, et al.** 1999 Maturational disturbance of chondrocytes in Cbfa1-deficient mice. Dev Dyn 214:279–290
7. **Kim IS, Otto F, Zabel B, Mundlos S** 1999 Regulation of chondrocyte differentiation by Cbfa1. Mech Dev 80:159–170
8. **Banerjee C, McCabe LR, Choi J-Y, et al.** 1997 Runt homology domain proteins in osteoblast differentiation: AML-3/CBFA1 is a major component of a bone specific complex. J Cell Biochem 66:1–8
9. **Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G** 1997 Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747–754
10. **Ferguson C, Alpern E, Miclau T, Helms JA** 1999 Does adult fracture repair recapitulate embryonic skeletal formation? Mech Dev 87:57–66
11. **Xiao ZS, Thomas R, Hinson TK, Quarles LD** 1998 Genomic structure and isoform expression of the mouse, rat and human Cbfa1/Osf2 transcription factor. Gene 214:187–197
12. **Pozner A, Goldenberg D, Negreanu V, et al.** 2000 Transcription-coupled translation control of AML1/RUNX1 is mediated by cap- and internal ribosome entry site-dependent mechanisms. Mol Cell Biol 20:2297–2307
13. **Drissi H, Luc Q, Shakoori R, et al.** 2000 Transcriptional autoregulation of the bone related CBFA1/RUNX2 gene. J Cell Physiol 184:341–350
14. **Stewart M, Terry A, Hu M, et al.** 1997 Proviral insertions induce the expression of bone-specific isoforms of PEBP2αA (CBFA1): evidence for a new myc collaborating oncogene. Proc Natl Acad Sci USA 94:8646–8651
15. **Mundlos S** 1999 Cleidocranial dysplasia: clinical and molecular genetics. J Med Genet 36:177–182
16. **Zhang YW, Yasui N, Ito K, et al.** 2000 A RUNX2/PEBP2α A/CBFA1 mutation displaying impaired transactivation and Smad interaction in cleidocranial dysplasia. Proc Natl Acad Sci USA 97:10549–10554
17. **Ogawa E, Maruyama M, Kagoshima H, et al.** 1993 PEBP2/PEA2 represents a family of transcription factors homologous to the products of the *Drosophila* runt gene and the human AML1 gene. Proc Natl Acad Sci USA 90:6859–6863
18. **Harada H, Tagashira S, Fujiwara M, et al.** 1999 Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem 274:6972–6978
19. **Levanon D, Negreanu V, Bernstein Y, Bar-Am I, Avivi L, Groner Y** 1994 AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. Genomics 23:425–432
20. **Satake M, Nomura S, Yamaguchi-Iwai Y, et al.** 1995 Expression of the runt domain-encoding PEBP2α genes in T cells during thymic development. Mol Cell Biol 15:1662–1670
21. **Tsuji K, Ito Y, Noda M** 1998 Expression of the PEBP2αA/AML3/CBFA1 gene is regulated by BMP4/7 heterodimer and its overexpression suppresses type I collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells. Bone 22:87–92
22. **Castagnola P, Gennari M, Gaggero A, et al.** 1996 Expression of runtB is modulated during chondrocyte differentiation. Exp Cell Res 223:215–226
23. **Thirunavukkarasu K, Mahajan M, McLarren KW, Stifani S, Karsenty G** 1998 Two domains unique to osteoblast-specific transcription factor Osf2/Cbfa1 contribute to its transactivation function and its inability to heterodimerize with Cbfβ. Mol Cell Biol 18:4197–4208
24. **Xiao ZS, Hinson TK, Quarles LD** 1999 Cbfa1 isoform overexpression up-regulates osteocalcin gene expression in non-osteoblastic and pre-osteoblastic cells. J Cell Biochem 74:596–605
25. **Javed A, Guo B, Hiebert S, et al.** 2000 Groucho/TLE/R-Esp proteins associate with the nuclear matrix and repress RUNX (CBFα/AML/PEBP2α) dependent activation of tissue-specific gene transcription. J Cell Sci 113:2221–2231
26. **Javed A, Barnes GL, Jassanya BO, et al.** 2001 *runt* homology domain transcription factors (Runx, Cbfa, and AML) mediate repression of the bone sialoprotein promoter: evidence for promoter context-dependent activity of Cbfa proteins. Mol Cell Biol 21:2891–2905
27. **Katagiri T, Yamaguchi A, Ikeda T, et al.** 1990 The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 172:295–299
28. **Yamaguchi A, Katagiri T, Ikeda T, et al.** 1991 Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113:681–687
29. **Gori F, Thomas T, Hicok KC, Spelsberg TC, Riggs BL** 1999 Differentiation of human marrow stromal precursor cells: bone morphogenetic protein-2 increases OSF2/CBFA1, enhances osteoblast commitment, and inhibits late adipocyte maturation. J Bone Miner Res 14:1522–1535
30. **Lee MH, Javed A, Kim HJ, et al.** 1999 Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor β1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. J Cell Biochem 73:114–125
31. **Owen TA, Aronow M, Shalhoub V, et al.** 1990 Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420–430
32. **Rosen V, Nove J, Song JJ, Thies RS, Cox K, Wozney JM** 1994 Responsiveness of clonal limb bud cell lines to bone morphogenetic protein 2 reveals a sequential relationship between cartilage and bone cell phenotypes. J Bone Miner Res 9:1759–1768
33. **Katagiri T, Yamaguchi A, Komaki M, et al.** 1994 Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766
34. **Yang R, Gerstenfeld LC** 1997 Structural analysis and characterization of tissue and hormonal responsive expression of the avian bone sialoprotein (BSP) gene. J Cell Biochem 64:77–93
35. **Ji C, Casinghino S, Chang DJ, et al.** 1998 CBFa(AML/PEBP2)-related elements in the TGF-β type I receptor promoter and expression with osteoblast differentiation. J Cell Biochem 69:353–363
36. **Frenkel B, Montecino M, Green J, et al.** 1996 Basal and vitamin D-responsive activity of the rat osteocalcin promoter in stably transfected osteosarcoma cells: requirement of upstream sequences for control by the proximal regulatory domain. Endocrinology 137:1080–1088
37. **Banerjee C, Stein JL, van Wijnen AJ, Frenkel B, Lian JB, Stein GS** 1996 Transforming growth factor-β1 responsiveness of the rat osteocalcin gene is mediated by an activator protein-1 binding site. Endocrinology 137:1991–2000
38. **Meyers S, Lenny N, Sun W-H, Hiebert SW** 1996 AML-2 is a potential target for transcriptional regulation by the t(8;21) and t(12;21) fusion proteins in acute leukemia. Oncogene 13:303–312
39. **Meyers S, Hiebert SW** 1995 Indirect and direct disruption of transcriptional regulation in cancer: E2F and AML-1. Crit Rev Eukaryot Gene Expr 5:365–383
40. **Lee KS, Kim HJ, Li QL, et al.** 2000 Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between runx2 and smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20:8783–8792
41. **Namba K, Abe M, Saito S, Satake M, Ohmoto T, Watanabe T, Sato Y** 2000 Indispensable role of the transcription factor PEBP2/CBF in angiogenic activity of a murine endothelial cell MSS31. Oncogene 19:106–114
42. **Ausubel FM, Brent R, Kingston RE, et al.** 1989 Current protocols in molecular biology. New York: Greene and Wiley
43. **Zeng C, van Wijnen AJ, Stein JL, et al.** 1997 Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBFα transcription factors. Proc Natl Acad Sci USA 94:6746–6751
44. **Choi J-Y, Lee B-H, Song K-B, et al.** 1996 Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3–E1 cells. J Cell Biochem 61:609–618
45. **Yamamoto N, Akiyama S, Katagiri T, Namiki M, Kurokawa T, Suda T** 1997 Smad1 and smad5 act downstream of intracellular signalings of BMP-2 that inhibits myogenic differentiation and induces osteoblast differentiation in C2C12 myoblasts. Biochem Biophys Res Commun 238:574–580
46. **Levanon D, Bernstein Y, Negreanu V, et al.** 1996 A large variety of alternatively spliced and differentially expressed mRNAs are encoded by the human acute myeloid leukemia gene AML1. DNA Cell Biol 15:175–185
47. **Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA** 1996 Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci USA 93:3444–3449
48. **Ogawa S, Harada H, Fujiwara M, Tagashira S, Katsumata T, Takada H** 2000

Cbfa1, an essential transcription factor for bone formation, is expressed in testis from the same promoter used in bone. DNA Res 7:181–185
49. **Bonham K, Ritchie SA, Dehm SM, Snyder K, Boyd FM** 2000 An alternative human SRC promoter and its regulation by hepatic nuclear factor (HNF)-1α. J Biol Chem 275:37604–37611
50. **Zernik J, Kream B, Twarog K** 1991 Tissue-specific and dexamethasone-inducible expression of alkaline phosphatase from alternative promoters of the rat bone/liver/kidney/placenta gene. Biochem Biophys Res Commun 176: 1149–1156
51. **Principaud E, Spohr G** 1991 Xenopus laevis c-myc I and II genes: molecular structure and developmental expression. Nucleic Acids Res 19:3081–3088
52. **Christiansen M, Kveiborg M, Kassem M, Clark BF, Rattan SI** 2000 CBFA1 and topoisomerase I mRNA levels decline during cellular aging of human trabecular osteoblasts. J Gerontol A Biol Sci Med Sci 55:B194–B200
53. **Xiao G, Jiang D, Thomas P, et al.** 2000 MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem 275: 4453–4459
54. **D'Souza RN, Aberg T, Gaikwad J, et al.** 1999 Cbfa1 is required for epithelial-mesenchymal interactions regulating tooth development in mice. Development 126:2911–2920
55. **Blyth K, Terry A, Mackay N, et al.** 2001 Runx2: a novel oncogenic effector revealed by in vivo complementation and retroviral tagging. Oncogene 20: 295–302
56. **Stewart K, Walsh S, Screen J, et al.** 1999 Further characterization of cells expressing STRO-1 in cultures of adult human bone marrow stromal cells. J Bone Miner Res 14:1345–1356
57. **Gronthos S, Zannettino AC, Graves SE, Ohta S, Hay SJ, Simmons PJ** 1999 Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. J Bone Miner Res 14:47–56
58. **Satomura K, Krebsbach P, Bianco P, Gehron RP** 2000 Osteogenic imprinting upstream of marrow stromal cell differentiation. J Cell Biochem 78:391–403
59. **Hicok KC, Thomas T, Gori F, Rickard DJ, Spelsberg TC, Riggs BL** 1998 Development and characterization of conditionally immortalized osteoblast precursor cell lines from human bone marrow stroma. J Bone Miner Res 13:205–217
60. **Ueta C, Iwamoto M, Kanatani N, et al.** 2001 Skeletal malformations caused by overexpression of Cbfa1 or its dominant negative form in chondrocytes. J Cell Biol 153:87–100
61. **Fleet JC, Hock JM** 1994 Identification of osteocalcin mRNA in nonosteoid tissue of rats and humans by reverse transcription-polymerase chain reaction. J Bone Miner Res 9:1565–1573
62. **Noda M, Yoon K, Thiede M, et al.** 1987 cDNA cloning of alkaline phosphatase from rat osteosarcoma (ROS 17/2.8) cells. J Bone Miner Res 2:161–164
63. **Qu Q, Perala-Heape M, Kapanen A, et al.** 1998 Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. Bone 22:201–209
64. **Dick A, Risau W, Drexler H** 1998 Expression of Smad1 and Smad2 during embryogenesis suggests a role in organ development. Dev Dyn 211:293–305
65. **Valdez MR, Richardson JA, Klein WH, Olson EN** 2000 Failure of Myf5 to support myogenic differentiation without myogenin, MyoD, and MRF4. Dev Biol 219:287–298

/THE LAST WORD

Another Anniversary for the War on Cancer

GERALD B. DERMER

In 1993, 526,000 Americans died of cancer—about 1400 people a day—even though conquering cancer became a national goal in December, 1971, now more than 22 years ago. Though tens of thousands of skilled scientists have been waging war against cancer in their laboratories for a generation, spending billions of tax, charitable, and investor dollars, the dread disease remains a metaphor for anything evil in society that spreads. The human statistics should be an issue of the most profound concern to the cancer industry because they are the only true measure of its performance, and the statistics remain very grim.

Why don't we have a cancer cure by now? The answer, in my opinion, is basic and essentially simple: The cell lines in which cancer is usually studied are unsuitable for the job. They do not mimic conditions in the human body.

The cancer industry has ignored the limitations of its most important piece of material—its favorite model—because "Nobody likes to ask if a model is really correct . . ."(Francis Crick, *What Mad Pursuit*, 1988, Basic Books, New York, p. 161). More than 40 years ago, these long-term cell cultures began their careers as stand-ins for real cancer based only on investigator faith in their reliability. Because they are so convenient for experimentation and the methods of molecular biology, cell lines today have become the standard for determining what cancer should be like. The facts indicate, however, that petri dish cancer is really a poor representation of malignancy, with characteristics profoundly different from the human disease.

When a normal or malignant body cell survives a crisis period and adapts to immortal life in culture, it takes an evolutionary-type step that enables the new line to thrive in its artificial environment. This step transforms a cell from one that is stable and differentiated to one that is not. Yet normal or malignant cells *in vivo* are not like that. This means that cell lines are really a new life form on Earth, neither human nor animal. Evidence of the contradictions between life on the bottom of a lab dish and in the body has been in the scientific literature for more than 30 years, evidence that has been systematically ignored by the cancer establishment.

Studies of human and animal cancer have shown that only differentiating, aging cells in organs are susceptible to cancer. Data from undifferentiated, ageless "normal" cell lines—like 3T3 in which the pathways that are struck by cancer, those of development and aging, are absent—cannot be relevant to cancer initiation in humans.

The widely disparate character of human tumor cell lines contributes greatly to chemotherapy's continued ineffectiveness against cancer. New drugs are selected for human trials because they kill tumor cell lines in the laboratory.

Surgical pathologists, the specialists who diagnose cancer, have long recognized that cancer cells are just misbehaving body cells. In other words, the immune system registers self when confronted by a malignant cell. This means that several decades of highly publicized, well-funded research on immunotherapy has produced only mice that are cured of their cell line tumors.

The standard approach of most cancer scientists to experimentation produces little of practical value because it is flawed. Typically, an observation is first made in culture, then the investigator turns to human cancer. If the observation is duplicated, papers are written about the significant find. But, if you look long and hard enough *in vivo* you will always find what you seek. For example, there may be a rare human tumor that is immunogenic, but this is just the exception that proves the rule. There are human tumors in which a proto-oncogene is mutated, but there are others of the same type in which it is not. What is significant in culture, for example immunotherapy's killing power or the transformation of 3T3 cells by a mutated proto-oncogene, simply does not have the same significance for cells *in vivo*. Instead of this approach, models that mimic the human body and the developmental pathways of human cells, both normal and malignant, should be first identified. Only then will truly significant observations be made.

How cancer is defined today depends on what courses have been taken, what books have been read, which journals have been studied, and what research training and practice have been followed. The result is confusion. An unnatural condition created in the laboratory is being mistaken for human cancer.

Every year, for as long as I can remember, cancer scientists and cancer physicians have met during the same week, under one roof, at overlapping conferences. They no longer will do so. This year, the American Association for Cancer Research and the American Association of Clinical Oncology begin meeting separately. The new meeting policy, initiated by the researchers, is as open an admission as one is likely to get from them that they really haven't been interested in the real world for a long time. ///

Gerald B. Dermer, a former cancer researcher, is the author of The Immortal Cell: Why Cancer Research Fails, *published by Avery Publishing Group, Garden City Park, NY. He can be reached at 1235 N. Sunnyvale, No. 87, Mesa, AZ 85205.*

The views expressed here are the author's own and not necessarily those of Bio/Technology.

CULTURE OF ANIMAL CELLS

A Manual of Basic Technique

R. Ian Freshney

Department of Clinical Oncology
Cancer Research Campaign Laboratories
University of Glasgow

Alan R. Liss, Inc., New York

Address all Inquiries to the Publisher
Alan R. Liss, Inc., 150 Fifth Avenue, New York, NY 10011

Printed in the United States of America.

Library of Congress Cataloging in Publication Data

Freshney, R. Ian.
Culture of animal cells.

Bibliography: p.
Includes index.
1. Tissue culture. 2. Cell culture. I. Title.
QH585.F74 1983 591'.07'24 82-24960
ISBN 0-8451-0223-0

Quantity

A major limitation of cell culture is the expenditure of effort and materials that goes into the production of relatively little tissue. A realistic maximum per batch for most small laboratories (2 or 3 people doing tissue culture) might be 1–10 g of cells. With a little more effort and the facilities of a larger laboratory, 10–100 g is possible; above 100 g implies industrial pilot plant scale, beyond the reach of most laboratories, but not impossible if special facilities are provided.

The cost of producing cells in culture is about ten times that of using animal tissue. Consequently, if large amounts of tissue (>10 g) are required, the reasons for providing them by tissue culture must be very compelling. For smaller amounts of tissue ($\leqslant 10$ g), the costs are more readily absorbed into routine expenditure; but it is always worth considering whether assays or preparative procedures can be scaled down. Semimicro- or micro-scale assays can often be quicker due to reduced manipulation times, volumes, centrifuge times, etc. and are often more readily automated (see under Microtitration, Chapter 19).

Instability

This is a major problem with many continuous cell lines resulting from their unstable aneuploid chromosomal constitution. Even with short-term cultures, although they may be genetically stable, the heterogeneity of the cell population, with regard to cell growth rate, can produce variability from one passage to the next. This will be dealt with in more detail in Chapters 12 and 18.

MAJOR DIFFERENCES *IN VITRO*

Many of the differences in cell behavior between cultured cells and their counterparts *in vivo* stem from the dissociation of cells from a three-dimensional geometry and their propagation on a two-dimensional substrate. Specific cell interactions characteristic of the histology of the tissue are lost, and, as the cells spread out, become mobile and, in many cases, start to proliferate, the growth fraction of the cell population increases. When a cell line forms it may represent only one or two cell types and many heterotypic interactions are lost.

The culture environment also lacks the several systemic components involved in homeostatic regulation *in vivo*, principally those of the nervous and endocrine systems. Without this control, cellular metabolism may be more constant *in vitro* than *in vivo*, but may not be truly representative of the tissue from which the cells were derived. Recognition of this fact has led to the inclusion of a number of different hormones in culture media (see Chapter 9) and it seems likely that this trend will continue.

Energy metabolism *in vitro* occurs largely by glycolysis, and although the citric acid cycle is still functional it plays a lesser role.

It is not difficult to find many more differences between the environmental conditions of a cell *in vitro* and *in vivo* and this has often led to tissue culture being regarded in a rather skeptical light. Although the existence of such differences cannot be denied, it must be emphasized that many specialized functions are expressed in culture and as long as the limits of the model are appreciated, it can become a very valuable tool.

Origin of Cells

If differentiated properties are lost, for whatever reason, it is difficult to relate the cultured cells to functional cells in the tissue from which they were derived. Stable markers are required for characterization (see Chapter 15); and in addition, the culture conditions may need to be modified so that these markers are expressed (see next chapter).

DEFINITIONS

There are three main methods of initiating a culture [Schaeffer, 1979] (see Glossary and Fig. 1.2): (1) *Organ culture* implies that the architecture characteristic of the tissue *in vivo* is retained, at least in part, in the culture. Toward this end, the tissue is cultured at the liquid/gas interface (on a raft, grid, or gel) which favors retention of a spherical or three-dimensional shape. (2) In *primary explant culture* a fragment of tissue is placed at a glass (or plastic)/liquid interface where, following attachment, migration is promoted in the plane of the solid substrate. (3) *Cell culture* implies that the tissue or outgrowth from the primary explant is dispersed (mechanically or enzymatically) into a cell suspension which may then be cultured as an adherent monolayer on a solid substrate, or as a suspension in the culture medium.

Organ cultures, because of the retention of cell interactions as found in the tissue from which the culture was derived, tend to retain the differentiated properties of that tissue. They do not grow rapidly (cell proliferation is limited to the periphery of the explant and is restricted mainly to embryonic tissue) and hence cannot

be propagated; each experiment requires fresh explantations and this implies greater effort and poorer sample reproducibility than with cell culture. Quantitation is, therefore, more difficult and the amount of material that may be cultured is limited by the dimensions of the explant ($\leqslant 1$ mm^3) and the effort required for dissection and setting up the culture.

However, it must be emphasized that organ cultures do retain specific histological cell interactions without which it may be difficult to reproduce the characteristic cell behavior of the tissue.

Cell cultures may be derived from primary explants or dispersed cell suspensions. Because cell proliferation is often found in such cultures, propagation of cell

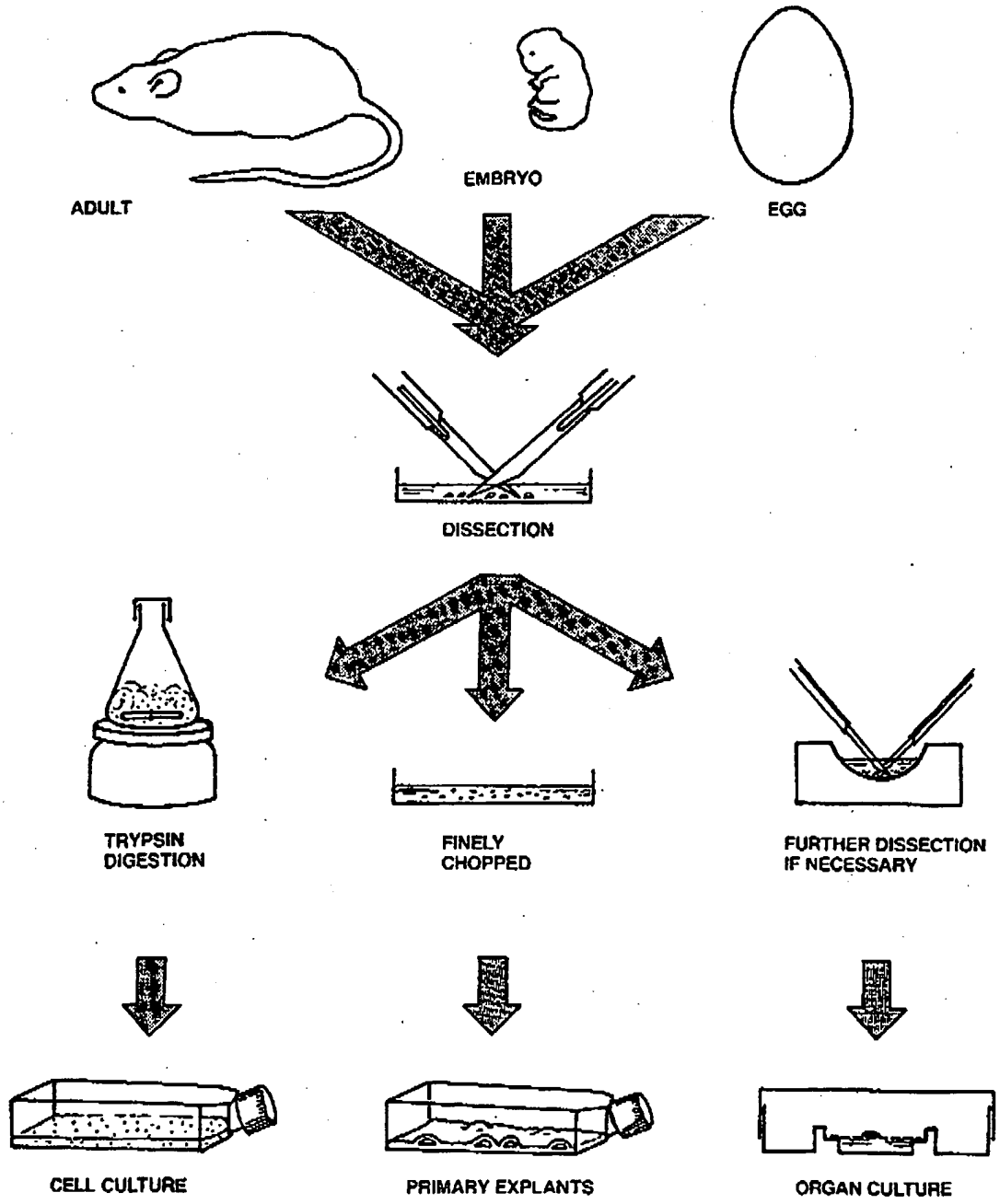


Fig. 1.2. *Types of tissue culture.*

lines becomes feasible. A monolayer or cell suspension, with a significant growth fraction, may be dispersed by enzymatic treatment or simple dilution and reseeded, or subcultured, into fresh vessels. This constitutes a "passage" and the daughter cultures so formed are the beginnings of a "cell line."

The formation of a cell line from a primary culture implies (1) an increase in total cell number over several generations, (2) that cells or cell lineages with similar high growth capacity will predominate, resulting in (3) a degree of uniformity in the cell population. The line may be characterized, and those characteristics will apply for most of its finite lifespan. The derivation of "continuous" (or "established" as they were once known) cell lines usually implies a phenotypic change or "transformation" and will be dealt with in Chapters 2 and 16.

When cells are selected from a culture, by cloning or some other method, the subline is known as a "cell strain." Detailed characterization is then implied. Cell lines may be propagated as an adherent monolayer or in suspension. *Monolayer* culture implies that adherence to the substrate is an integral part of survival and subsequent cell proliferation and is the mode of culture common to most normal cells with the exception of mature hemopoietic cells. *Suspension* cultures are derived from cells which can survive and proliferate without attachment; and this property seems to be reserved for hemopoietic cells, transformed cell lines, or cells from malignant tumors. It can be shown, however, that a small proportion of cells exists in many normal tissues, capable of proliferation in suspension (see Chapter 16). The identity of these cells remains unclear, but a relationship to the stem cell or uncommitted precursor cell compartment has been postulated. This concept implies that some cultured cells represent precursor pools within the tissue of origin, and the generality of this observation will be discussed more fully in the next chapter. Are cultured cell lines more representative of precursor cell compartments *in vivo* than of fully differentiated cells, bearing in mind that most differentiated cells do not normally divide?

Because they may be propagated as a uniform cell suspension or monolayer, cell cultures have many advantages in quantitation, characterization, and replicate sampling, but lack the potential for cell-cell interaction and cell-matrix interaction afforded by organ cultures. For this reason many workers have attempted to reconstitute three-dimensional cellular structures using aggregated cell suspension ("spheroids") or perfused high-density cultures (such as Vitafiber, Amicon) (see Chapter 21). In many ways some of the more exciting developments in tissue culture arise from recognizing the necessity of specific cell interaction in homogeneous or heterogeneous cell populations in culture. This may mark the transition from an era of fundamental molecular biology, where the regulatory processes have been worked out at the cellular level, to an era of cell or tissue biology where this understanding is applied to integrated populations of cells.

Current Treatment Options for the Restoration of Articular Cartilage

James E. Gilbert, MD

INTRODUCTION

Hyaline cartilage, as found in normal joints, has an important role in decreasing friction and mechanical load. Its unique biochemical and histological structure is inextricably linked to its biomechanical function of protecting the underlying subchondral bone. Understandably, when hyaline cartilage breaks down, changes occur in the subchondral bone, which are manifested by radiographic changes and the clinical symptoms of osteoarthritis.

Recent advances in the basic sciences, with an increased understanding of the structure and function of articular cartilage and the mechanisms by which articular cartilage breaks down, have opened up a new field where the possibility now exists for restoring articular surfaces. We now know from clinical and basic science research that lesions once thought to be irreparable may be repairable. Current techniques are successful to a certain degree in producing repair tissue, but as of yet, there is no single technique that is capable of reduplicating the histological structure and biomechanical function of hyaline cartilage. The extent to which the repair tissue approximates the normal histological structure of hyaline cartilage can be correlated to its ultimate ability to resist mechanical load and maintain good clinical function with pain relief.

The dilemma in restoring articular surfaces appears to lie with the chondrocytes. The chondrocytes are the metabolic power plants that produce the extracellular matrix. Once chondrocytes mature or differentiate, their capacity to reproduce slows down. They carry on their metabolic activities, embedded in lacunae within the extracellular matrix they are producing. Although isolated within this self-made milieu, the chondrocytes can respond to growth factors, cytokines, and mechanical influences produced externally from the environment.[4,9] Changes in these stimulators of chondrocytes have been shown to have profound effects on the degeneration and synthesis of articular cartilage.[4,9] Chondrocytes cultured from the articular cartilage of young individuals are more sensitive to these stimulators and are able to maintain an increased capacity to divide and expand.[4,9]

The symphony that the chondrocytes orchestrate is a fine balance between synthesis and degradation. Like all biological structures, there exists a natural turnover process that maintains the articular cartilage in its optimal biomechanical states. For whatever reason, be it genetic or trauma, when this delicate balance is disrupted, it is tipped toward degradation.[4,9] Early on in this process, synthesis may still keep pace, but if the condition that initiated the degenerative process continues unabated, synthesis can no longer keep up and the patient becomes symptomatic.

Osteoarthritis is a degenerative process that occurs over time. While its etiology may be multifactorial, the final common pathway is a shift in this balance from synthesis to degradation. Recently, interleukin-1 has been implicated as a key component in the pathogenesis of osteoarthritis. It appears that interleukin-1 can stimulate protein modulators known as metalloproteinases.[9] Metalloproteinases have been implicated in the break-

Dr Gilbert is from the Department of Orthopedic Surgery, Baylor University Medical Center, Dallas, Texas.

Reprint requests: James E. Gilbert, MD, Dept of Orthopedic Surgery, Baylor University Medical Ctr, 3500 Gaston Ave, Dallas, TX 75246.

down of proteoglycans and collagen in the extracellular matrix. There is currently a great deal of interest in regulating these metalloproteinases to prevent the progression of osteoarthritis. Tissue inhibitors of metalloproteinases have been described and have been shown to counteract some of the degradative properties of metalloproteinases.[9]

Studies have shown that following injury to articular cartilage, an initial decrease in collagen and proteoglycan content is followed by a subsequent increase. This apparent compensatory synthetic response by the chondrocytes, however, is usually ineffective. There is disaggregation of the proteoglycan aggregates and also disorganization of the collagen fibrillar network. This allows for an increase in the permeability of the extracellular matrix and an increase in tissue hydration. The collagen and proteoglycan fibrillar meshwork, which is now disrupted, cannot maintain an effective swelling pressure, and the overall stiffness of the articular cartilage is dramatically reduced. Eventually, the synthetic machinery of the chondrocytes is smothered as the articular cartilage is overloaded. The production of collagen and proteoglycans late in the degenerative process thus is significantly diminished.[9]

The barrier that maintains the privileged environment of the chondrocyte may be the tidemark. The tidemark is a histologically defined layer in articular cartilage below which cartilage becomes calcified and then ossified to become subchondral bone. It may serve as a selective barrier. As individuals mature through adolescence, the articular cartilage looses some of its regenerative capacity and plasticity as the tidemark becomes more defined. Fractures that extend below the tidemark have a greater potential to heal because they are exposed to the vascular supply of the subchondral bone. A fibrin clot can form, and mesenchymal precursor cells present are stimulated to regenerate fibrocartilage.

Unfortunately, fibrocartilage is a reparative tissue that consists of a mixture of type I and type II collagen. It lacks the unique zonal organization of hyaline cartilage. It thus lacks durability and breaks down over time. Any repair tissue must therefore receive the appropriate stimulatory signals to reduplicate the normal histology of hyaline cartilage to maintain normal biological function. Ultimately, an effective reparative process will involve responsive chondrocytes receiving appropriate signals in a conducive environment.

NONOPERATIVE MEDICAL TREATMENT

In most instances, the breakdown of articular cartilage is a multifactorial event. There is a final common pathway, however, and the etiology can be traced to a mechanical overload of the chondrocytes and a weakened extracellular matrix. Thus, methods that unload the joint can tip the balance from articular cartilage degeneration to articular cartilage synthesis. Exactly how the chondrocyte perceives a reduction in mechanical load is not clear, but it has been shown that with reduction in mechanical load, the chondrocyte can increase its metabolic activity.[9] Therefore, simple measures, such as weight reduction, physical therapy to increase the dynamic muscular support around joints, and braces and orthotics that unload the joint, can to some extent allow the chondrocyte to rejuvenate its synthetic machinery. With the regeneration of articular cartilage, there is an upregulation of the ribosomes, endoplasmic reticulum, DNA, and mRNA, which are involved in collagen and matrix production.[4]

Several medications are currently being used to treat osteoarthritis. The efficacy of nonsteroidal anti-inflammatory drugs (NSAIDS) in noninflammatory arthropathies such as osteoarthritis has not been shown clinically to be any better than simple analgesics such as acetaminophen. Oral chondroitin sulfates derived from shark cartilage and oral glycosaminoglycan precursors similarly have not been shown in credible randomized, prospective studies to be any better than placebos. Hyaluronic acid derivatives recently have been approved by the FDA for injection into synovial joints. The problem is that multiple injections are required, with an increased number of office visits, increased cost, and diminishing results over time. Furthermore, it seems scientifically implausible that after the ingestion of a protein in the gastrointestinal tract there can be reconstitution of a biologically active precursor that helps to promote joint lubrication.

ARTHROSCOPIC LAVAGE AND DEBRIDEMENT

Exactly how arthroscopic lavage and debridement may help the early symptoms of osteoarthritis is not entirely clear at this time. Many researchers have theorized that the benefit is derived from removing harmful enzymes such as metalloproteinases from the joint that have been shown to perpetuate the vicious cycle of breakdown.[7] In early stages, removing these degradative enzymes from the joint may allow the chondrocyte to upregulate its biosynthetic activity.

Another mechanism by which lavage may relieve the symptoms of osteoarthritis and increase the resiliency and stiffness of articular cartilage is through changing the ionic environment within the synovial fluid. The articular cartilage maintains a swelling pressure as the negatively charged proteoglycans attract positively charged sodium ions. In turn, the sodium ions create an osmotic effect that increases the permeability of the articular cartilage. In the early stages, in which the collagen fibril complex is not yet disorganized, maintaining the swelling pressure by lavage can help to maintain the stiffness of the articular cartilage and help resist load.

When the articular cartilage breakdown has progressed to the later stages of osteoarthritis, such as in bone-on-bone articulation, the beneficial effect of lavage obviously will be minimal. In addition, if predisposing malalignment is not corrected, the beneficial effects seem to be minimized.

Why mechanical debridement is of benefit above and beyond the lavage process alone is also poorly understood. Debridement of loose articular cartilage flaps obviously relieves mechanical symptoms and also may prevent further delamination. It is also possible that removal of loose pieces of articular cartilage may decrease the load of degradative proteolytic enzymes, and the removal of fibrillated and delaminating cartilage (shaving) may improve joint loading and mechanics.

MARROW STIMULATION TECHNIQUES

There are several techniques that use stimulation of the subchondral bone marrow to produce a type of repair cartilage known as "fibrocartilage." Abrasion arthroplasty, subchondral drilling, and microfracture are all techniques that expose the subchondral bone and produce a fibrin clot. In the fibrin clot, there are primitive undifferentiated mesenchymal stem cells that are capable of differentiating into either bone or cartilage. The differentiation is influenced by the presence of various biological growth factors and also environmental and mechanical factors.

Unfortunately, the fibrocartilage repair tissue, which contains predominantly type II collagen, is fragile. The fibrocartilage produced must be protected from excessive load. This sometimes requires prolonged restricted weightbearing or unloading osteotomies. In the end, most studies show that good and excellent results are only maintained for short periods of time.[8]

Abrasion Arthroplasty

Abrasion arthroplasty involves superficially abrading the subchondral bone so that bleeding and a fibrin clot are produced. It is also thought that debriding the degenerative articular cartilage back to normal tissues is important so that the fibrin clot is capable of binding. In 399 patients who underwent arthroscopy and abrasion arthroplasty, Johnson[8] noted that only 12% of patients had no complaints. Histologically, the repair tissue is initially fibrous and becomes fibrocartilage in about 4 to 6 months. Characterization of the collagen shows increasing amounts of type II collagen with smaller amounts of type I collagen. The repair tissue continues to remodel up to 2 years postoperatively.

Chondrocytes have shown age-related changes with a limited capacity to divide as they get older. This characteristic may explain the slightly better results from various marrow stimulation techniques in younger patients. Friedman et al[5] found that among patients who averaged 54 years of age, 53% exhibited some improvement, whereas in patients who averaged 40 years old, 86% showed improvement. This study further illustrates the importance of demographics and age in any study reporting the outcome of articular cartilage restoration techniques.

Subchondral Drilling

First described by Pridie in 1956, subchondral drilling is similar to abrasion arthroplasty except that the subchondral bone is penetrated in a more precise pinpoint fashion using small drills. Like abrasion arthroplasty, a fibrin clot is produced. Undifferentiated mesenchymal stem cells will fill the defect with a mixture of various repair tissues. The end result is once again some degree of fibrocartilage. In one study by Tippett,[12] a group of patients who underwent subchondral drilling and high tibial valgus osteotomy showed superior results to those receiving osteotomy alone. This further illustrates the importance of unloading damaged or repaired articular cartilage.

Microfracture

This technique, popularized by Steadman, is similar to abrasion arthroplasty and subchondral drilling. Small pinpoint lesions made by picks in the subchondral bone produce bleeding and a fibrin clot. Abnormal degenerative cartilage needs to be debrided back to a healthy rim of cartilage. Although many patients have undergone microfracture techniques, there are no large studies revealing outcome.

Most rehabilitation protocols after marrow stimulation techniques call for some form of protective weightbearing and continuous passive motion. In addition, it is believed that significant predisposing fractures such as malalignment or obesity need to be corrected to obtain better results. This allows redistribution of load and protects the fragile fibrocartilage that is produced.

PERIOSTEAL AND PERICHONDRAL GRAFTS

These techniques involve soft-tissue interposition grafts placed into traumatic, debrided, or resected articular cartilage defects. Of most promise have been the periosteal and perichondral grafts. Like bone marrow stimulation techniques, age, motion, and protection of the repair tissue appears to be important. Periosteal and perichondral grafts appear to provide a source of undifferentiated cells. Given the correct growth factors and microenvironment, they can produce "hyaline-like" cartilage. In the case of periosteum, the cells appear to be derived from the cambium layer, which is the layer adjacent to the bone. In addition to being a relatively abun-

dant tissue and thus a source of precursor cells, the periosteum or perichondrium also may act as a matrix and also afford the host precursors cells some protection from excessive loading. Whether the growth factors are derived from the soft-tissue graft or come from the adjacent articular cartilage or the synovium has not been determined.

Various animal studies and clinical experience have shown that perichondral and periosteal grafts placed in articular cartilage defects can produce hyaline-like cartilage.[10] Unfortunately, with time, the interposition grafts undergo endochondral ossification. This leads to break down and poor long-term results. The presence of type X collagen heralds the onset of endochondral ossification. Motion, normal loading, and younger age appear to give better results by minimizing the amount of endochondral ossification. Thus, while interposition grafts appear promising, the potential to differentiate into noncartilaginous pathways still exists and has not been solved.

CHONDROCYTE-STIMULATING FACTORS

Basic science research has identified a multitude of polypeptide growth factors. Bone morphogenic proteins, insulin such as growth factor, fibroblast growth factor, platelet-derived growth factor, and transforming growth factor beta (transforming growth factor-β) all have been shown to influence chondrocytes and other mesenchymal cells. They appear to affect the type of matrix synthesized, the degree of differentiation, proliferation, and migration. The chondrocyte-stimulating factors appear to mediate their effects through cell surface receptors on the chondrocytes, which are known as integrins. These factors then may directly modify the extracellular matrix.

In addition, environmental and mechanical factors such as changes in stress, strains, pressure, and flow seem to produce changes in the concentrations of these factors.[6] Sellers et al[11] recently demonstrated that recombinant human bone morphogenic protein-2 (rhBMP-2) can accelerate the healing of full-thickness defects of articular cartilage.

While chondrocytes respond in various ways to the growth factors, there is currently a great interest in the implantation of chondrocytes or mesenchymal stem cells into chondral defects, along with various chondrogenic-stimulating factors. If cartilage-producing cells can be placed into chondral defects and then given the right clues with chondrogenic-stimulating factors, it is hoped that the cartilage produced will be more likely to resemble hyaline cartilage. This is currently the theory behind autologous chondrocyte implantation techniques.

AUTOLOGOUS CHONDROCYTE IMPLANTATION

The technique of autologous chondrocyte implantation has received a great deal of recent attention in the press and in the scientific literature.[3] Cultured chondrocytes that are harvested from the patient are reimplanted after 3 to 4 weeks of culturing. At implantation, the osteochondral defect is debrided of all fibrous or damaged tissue, and a periosteal flap is meticulously sewn to the edge of normal articular cartilage. The cultured chondrocytes are then placed underneath the periosteal flap, and the flap is sealed.

It is important that the cambium layer of the periosteum is facing the joint as it may serve as a source of growth factors or cells for the new matrix. The cultured chondrocytes begin to produce an extracellular matrix that closely resembles hyaline cartilage. Histological biopsies, however, do reveal that there is still some disorganization of the chondrocytes and collagen matrix that is not seen in normal hyaline cartilage. Thus, the repaired tissue only can be called hyaline-like articular cartilage.

One of the main concerns about the autologous chondrocyte implantation technique is the expense. In addition, has not been shown that the natural history of a patient receiving an autologous chondrocyte implantation is any better than that of a patient who develops fibrocartilage after a marrow-stimulating technique. Recently, the results of chondrocyte implantation in cartilage defects in rabbits could not be reproduced in the canine model.[2] The reasons remain unclear. Obviously, prospective randomized studies comparing the hyaline-like tissue produced in autologous chondrocyte implantation with that of the fibrocartilage tissue produced in other techniques are needed.

SYNTHETIC MATRICES

Synthetic matrices are being developed out of collagen, carbon fibers, and glycosaminoglycan gels. They serve as a scaffolding or framework that helps to promote cell migration and proliferation. It is hoped that they can be used to fill articular cartilage defects and therefore promote the regeneration of a more normal joint contour.

OSTEOCHONDRAL AUTOGRAFTS

These techniques avoid the problems encountered with differentiation of stem cells into pathways of endochondral ossification and fibrocartilage production by grafting full-thickness plugs of subchondral bone and hyaline cartilage into defects. Single and multiple plugs can be placed into appropriate osteochondral defects. When multiple plugs are placed, the technique is known as mosaicplasty.

Defects >2 cm are difficult to fill using these techniques because it is difficult to contour the plugs to the

articular surface. Also, the interface between the donor and recipient hyaline cartilage is a cleft that usually is filled with fibrocartilage. While several studies have shown encouraging results with these techniques, the long-term biomechanical strength of these plugs has yet to be determined.[1]

SUMMARY

Over the past several decades, much has been learned about articular cartilage and its physiological capacity to restore itself. While articular cartilage does appear to have some regenerative capabilities, it appears to lose this capacity over a period of time, making restoration of articular surfaces more and more difficult. To date, no technique has been completely successful in achieving exactly normal regenerative articular cartilage.

Arthroscopic lavage and debridement provides temporary relief of symptoms. This probably works by removing degradative enzymes that contribute to synovitis and also to the further breakdown of articular cartilage.

Bone marrow stimulation techniques such as abrasion arthroplasty, drilling, and microfracture produce only fibrocartilage and therefore do not offer a long-term cure. Perichondral and periosteal interposition grafts produce repair tissue that is similar to hyaline cartilage but also lack the mechanical durability. Like bone marrow stimulation techniques, interposition grafts introduce precursor cells, which have a tendency to differentiate along lines other than cartilage. This leads to an inferior quality of repair tissue.

Currently, chondrogenic-stimulating factors and artificial matrices are currently being researched and developed. Much has been learned about the various growth factors that stimulate chondrocyte differentiation and extracellular matrix production, but to date, there has not been a clinical technique that has shown any long-term promise. Ultimately, the goal will be to take precursor cells from an easily accessible source such as the iliac crest, mix them with growth factors that have been derived genetically in the lab, and provide an artificial matrix that in combination can produce restoration of articular cartilage at minimal cost and patient morbidity.

Autologous osteochondral transplant systems have shown encouraging results but there are still problems. Graft matching and contouring to the recipient articular surface is difficult. Donor sites can be a limiting factor. Furthermore, the fibrocartilaginous interface between the donor and recipient site may contribute to breakdown in the long run.

Autologous chondrocyte implantation is a biological repair process that also has shown encouraging results. It must be remembered that this is not normal articular cartilage—it is only hyaline-like cartilage. The technique is expensive and is technically difficult to perform. There are no randomized prospective studies that compare the natural history of the repair tissue to that of other forms of repair tissue. Long-term functional outcome is still a significant question mark. In addition, it has not been shown that autologous chondrocyte implantation can prevent degenerative changes.

In the future, we probably will see delivery systems using stimulating growth factors, chondrocytes, and synthetically derived matrices. When placed in combination and with the right mechanical stimuli, we may ultimately achieve true restoration of articular cartilage.

REFERENCES

1. Bobic V. Arthroscopic osteochondral autograft transplantation in anterior cruciate ligament reconstruction—a preliminary clinical study. Knee surgery, sports traumatology. *Arthroscopy*. 1996;3:262-264.
2. Breinan HA, Minas T, Hsu HP, Nehrer S, Sledge CB, Spector M. Effect of cultured autologous chondrocytes on repair of chondral defects in a canine model. *J Bone Joint Surg Am*. 1997;79:1439-1451.
3. Brittberg M, Lindahl A, Nilsson A, et al. Treatment of deep cartilage defects in the knee with autologous chondrocyte implantation. *N Engl J Med*. 1994;331:889-895.
4. Buckwalter JA, Mow VC. Cartilage repair in osteoarthritis. In: Moskowitz RW, Howell DS, Goldbert VM, Mankin HJ, eds. *Osteoarthritis: Diagnosis and Medical/Surgical Management*. 2nd ed. Philadelphia, Pa: WB Saunders Co; 1992:71-107.
5. Friedman MD, Beras CC, Fox JM, et al. Preliminary results with abrasion arthroplasty in the osteoarthritic knee. *Clin Orthop*. 1984;182:200-205.
6. Homminga GN, Bulstra SK, Bouwmeester PSM, VanDerLinden AJ. Periochondral grafting for cartilage lesions of the knee. *J Bone Joint Surg Br*. 1990;72:1003-1007.
7. Howell DS, Treadwell BV, Trippel SB. Etiopathogenesis of osteoarthritis. In: Moskowitz RW, Howell DS, Goldberg VM, Mankin HJ, eds. *Osteoarthritis: Diagnosis and Medical/Surgical Management*. 2nd ed. Philadelphia, Pa: WB Saunders Co; 1992:233-252.
8. Johnson LL. Arthroscopic abrasion arthroplasty. In: McGinty JB, ed. *Operative Arthroscopy*. New York, NY: Raven Press; 1991:341-360.
9. Mankin MJ, Mow VC, Buckwalter JA, et al. Form and function of articular cartilage. In: Simon SR, ed. *Orthopaedic Basic Science*. Rosemont, Ill: American Academy of Orthopaedic Surgeons; 1994:1-44.
10. O'Driscoll SW, Keeley FW, Salter RB, et al. Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous passive motion. *J Bone Joint Surg Am*. 1988;70:595-606.
11. Sellers RS, Peluso D, Morris EA. The effect of recombinant human bone morphogenic protein-2 (rhBMP-2) on the healing of full-thickness defects of articular cartilage. *J Bone Joint Surg Am*. 1997;79:1452-1463.
12. Tippet JW. Articular cartilage drilling and osteotomy in osteoarthritis of the knee. In: McGinty JB, ed. *Operative Arthroscopy*. New York, NY: Raven Press; 1991:325-339.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 3 : A61K 37/00	A1	(11) International Publication Number: WO 84/ 01106
		(43) International Publication Date: 29 March 1984 (29.03.84)

(21) International Application Number: PCT/US83/01460

(22) International Filing Date: 23 September 1983 (23.09.83)

(31) Priority Application Numbers: 423,203
468,590
500,833

(32) Priority Dates: 24 September 1982 (24.09.82)
22 February 1983 (22.02.83)
3 June 1983 (03.06.83)

(33) Priority Country: US

(71) Applicant: UNITED STATES OF AMERICA represented by the SECRETARY UNITED SATES DEPARTMENT OF COMMERCE [US/US]; 5285 Port Royal Road, Springfield, VA 22161 (US).

(72) Inventors: SPORN, Michael, Benjamin ; 6630 Marywood Road, Bethesda, MD 20817 (US). ROBERTS, Anita, Bauer ; 9919 Harrogate Road, Bethesda, MD 20817 (US).

(74) Agents: KOCH, Robert, J. et al.; Schwartz, Jeffery, Schwaab, Mack, Blumenthal & Koch, 111 North Alfred Street, Post Office Box 299, Alexandria, VA 22314 (US).

(81) Designated States: AU, JP.

Published
With international search report.

(54) Title: REPAIR OF TISSUE IN ANIMALS

(57) Abstract

A composition for the promotion of cell proliferation and tissue repair in animals having as active ingredients a TGF-β which is activated by either a TGF-α or an EGF or both; and methods for administration. As another embodiment these active ingredients can be admixed with other (secondary) growth factors.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**LI**	Liechtenstein
AU	Australia	**LK**	Sri Lanka
BE	Belgium	**LU**	Luxembourg
BR	Brazil	**MC**	Monaco
CF	Central African Republic	**MG**	Madagascar
CG	Congo	**MR**	Mauritania
CH	Switzerland	**MW**	Malawi
CM	Cameroon	**NL**	Netherlands
DE	Germany, Federal Republic of	**NO**	Norway
DK	Denmark	**RO**	Romania
FI	Finland	**SE**	Sweden
FR	France	**SN**	Senegal
GA	Gabon	**SU**	Soviet Union
GB	United Kingdom	**TD**	Chad
HU	Hungary	**TG**	Togo
JP	Japan	**US**	United States of America
KP	Democratic People's Republic of Korea		

REPAIR OF TISSUE IN ANIMALS

This is a continuation-in-part application of USSN 06/468,590, filed 22 February 1983 (now pending), which is in turn a continuation-in-part application of USSN 06/423,203, filed 24 September 1982 (now abandoned).

BACKGROUND OF THE INVENTION

1. Field Of The Invention

This invention relates to compositions which promote repair of tissue, particularly fibroblast cells, in animals, particularly human beings. This invention further relates to a method of treating wounds by the topical or systemic administration of the compositions.

2. Description Of The Prior Art

There is a continuing need for the promotion of rapid cell proliferation at the site of wounds, burns, diabetic and decubitus ulcers, and other traumata.

A number of "growth factors" are known, which promote the rapid growth of animal cells. These growth factors include epidermal growth factor (EGF), transforming growth factors (TGF's), and nerve growth factor (NGF). However, prior to this invention, none of these growth factors have been found to be pharmaceutically acceptable agents for the acceleration of wound healing.

It has been shown that the mitogenic activity of insulin (a hormone) can be increased many-fold by the presence of prostaglandin $F_{2\alpha}$ (not exactly a hormone, but having similar properties - it causes constriction of vascular smooth muscle), see L. Jimenez de Asua, et al., Cold Spring Harbor Conf. Cell Proliferation Vol. 6, Sato, ed., Cold Spring Harbor Labs., New York, (1979) at Pp. 403-424. Similar activation of insulin has been reported with fibroblast growth factor by P. S. Rudland, et al., Proc. Natl. Acad. Sci., U.S.A., 71:2600-2604 (1974) and with EGF by R. W. Holley, et al., Proc. Natl. Acad. Sci., U.S.A., 71:2908-2911 (1974). Furthermore, in the "competance-progression" scheme of C. D. Stiles, et al., in Proc. Natl. Acad. Sci., U.S.A., 76:1279-1283 (1979), positive effects on cell growth have been demonstrated for platelet-derived growth factor or fibroblast growth factor in combination with members of the insulin family such as somatomedins A and C, the insulin-like growth factors.

Many new peptide growth factors have been isolated and characterized recently, as indicated in Tissue Growth Factors, R. Baserga, ed., Springer-Verlag pub., New York (1981), however there have been few studies on the activity of these materials in vivo. In many cases, the relatively small amounts of peptides available have limited the ability to study their properties in vivo. An important area for potential application of peptide growth factors is the enhancement of wound healing. Despite the need for rapid wound healing in the treatment of severe burns, trauma, diabetic and

decubitus ulcers, and many other conditions, at present there is no practical way to accelerate wound healing with pharmacological agents. Although it is suggested in Tissue Growth Factors, supra, at p. 123 that EGF might be of benefit in this area, it has yet to be extensively used in a practical way for wound healing.

SUMMARY OF THE INVENTION

This invention affords compositions for the promotion of cell proliferation in animals, especially fibroblast cells in human beings. The compositions have as their active ingredients, beta-type transforming growth factor (TGF-β) and an activating agent. The activating agents of this invention are selected from at least one of epidermal growth factor (EGF) and alpha-type transforming growth factor (TGF-α).

The TGF-β and the activating agent are preferably present in about equimolar amounts, and the active ingredients are present in an amount at least sufficient to stimulate cell proliferation (tissue repair).

As another embodiment, the activated TGF-β compositions of this invention may be admixed with other (secondary) growth factors to enhance their activity.

The compositions may be formulated in any suitable carrier for topical application, such as physiological saline solution or purified collagen suspension. The compositions also may be formulated in any suitable carrier for systemic administration.

The method of topical administration of the compositions of this invention is by direct application to a burn, wound, or other traumata situs. Periodic or continual further administration may be preferably indicated in most instances, since the active ingredients are physiologically utilized by the cells whose growth is being stimulated.

As a further embodiment, the compositions of this invention may be administered systemically by injection, enterally, transdermal patches, and the like, depending upon the nature and site of the traumata to be treated.

DETAILED DESCRIPTION OF THE INVENTION

The term, "transforming growth factor" (TGF) has been defined to include the set of polypeptides which confer the transformed phenotype on untransformed indicator cells. The transformed phenotype is operationally defined by the loss of density-dependent inhibition of growth in monolayer, overgrowth in monolayer, characteristic change in cellular morphology, and acquisition of anchorage independence, with the resultant ability to grow in soft agar. Untransformed, non-neoplastic cells will not form progressively growing colonies in soft agar, while the property of anchorage-independent growth of cells in culture has a particularly high correlation with neoplastic growth in vivo.

Although TGFs were first discovered in the conditioned medium of virally-transformed neoplastic mouse cells, the application of the acid/ethanol method for extraction of peptides from tissues has now shown that TGFs can be found in almost all tissues, both neoplastic and non-neoplastic, from all species of animals that have been examined thus far. Although TGF activity is usually measured with an in vitro phenotypic transformation assay, this does not imply that TGF

activity in vivo is necessarily related to the development of malignancy. Indeed, the transformed phenotype is a physiological state associated with normal embryological development, and transforming (onc) genes have been found in normal cells of essentially all vertebrates. The function of these onc genes from normal cells is not known at present. While there may be irreversible and excessive expression of TGF activity during malignant cell growth, the data at hand indicate that TGFs have a more benign and perhaps essential role in the function of normal cells. At present, it is not known what the intrinsic physiological roles of TGFs are. In this respect, TGFs are like many other peptide hormones or hormone-like agents which have recently been discovered and isolated; this is particularly true for many peptides of the nervous system, for which a defined chemical structure may be known, yet whose physiology is still a matter of uncertainty.

The initial description of sarcoma growth factor (SGF), the first of the TGFs to be isolated, was an important finding in tumor cell biology since it provided a direct mechanism for the expression of the neoplastic phenotype in a virally-transformed cell. Two important properties of SGF were described in these earliest studies, namely (1) that the effects of SGF in causing phenotypic transformation were dependent on its continued presence, and that these effects were reversible when SGF was removed, and (2) that the effects of SGF could be expressed in the very same cells that synthesized this peptide, a property that has been termed autocrine secretion. Although these two properties have not been definitively shown for all of the other more newly discovered TGFs, the functions of the entire set of TGFs can most reasonably

be assumed to be that of local, hormone-like agents that reversibly control cell function by paracrine or autocrine mechanisms.

Since the discovery of SGF in 1978, many TGFs have been described from diverse sources. These TGFs can be categorized into two groups: extracellular TGFs isolated from conditioned media of cultured cells, and intracellular (cell-associated) TGFs isolated by direct extraction of cells or tissues. Although extracellular TGFs have recently been isolated from non-neoplastic murine cells, use of conditioned medium has, in general, been restricted to neoplastic cell lines that could be grown in long-term, large-scale culture, including certain virally and chemically-transformed rodent cells and human tumor cell lines. The adaptation of an acid/ethanol extraction procedure to TGF isolation removed all limitations on cell types and quantities of tissues that could be examined. Using this procedure, extracts of all tissues or cells, whether of neoplastic or non-neoplastic origin, whether from adult or embryonic tissue, whether from human, bovine, or murine genomes, have been shown to promote colony formation in a soft agar assay; hence, by definition, these extracts contain TGF activity.

A variety of both epithelial and fibroblastic cell lines form colonies in soft agar in the presence of TGFs. However, the most commonly used indicator cell line is the rat kidney fibroblast cell clone, NRK 49F, which has been selected for its strong colony-forming response to the TGFs. Rat-1 cells and mouse AKR-2B cells have also been used successfully as indicator cells.

All of the TGFs referred to above are low molecular weight polypeptides which share with SGF the physical properties of acid and heat stability and sensitivity to treatment with both trypsin and dithiothreitol. However, there are marked differences in the biological properties of these TGFs, particularly with respect to their relationship to EGF. Certain TGFs, though antigenically distinct from EGF, have some structural homology to EGF, since they compete with EGF for receptor binding. Other TGFs do not compete with EGF for receptor binding, but instead are dependent on EGF for activity in the soft agar assay for colony formation. To remove the ambiguities implicit in the assignment of the general term "TGF" to these different factors, an operational classification of the members of the TGF family based on their interactions with EGF is suggested, both with respect to competition for binding to the EGF receptor and to the requirement for EGF for induction of colonies in soft agar.

As defined for purposes of this invention, TGF-α are those TGFs which compete with EGF for receptor binding and which do not require EGF for the induction of colony formation in soft agar. TGFs with these properties include SGF and other TGFs derived from neoplastic cells, as well as some TGFs from mouse embryos.

As defined for purposes of this invention, TGF-β are those TGFs which do not compete with EGF for receptor binding and which require EGF for the induction of colony growth in soft agar. When assayed in the presence of EGF, TGF-β represents the major colony-forming activity of the intracellular TGFs of both neoplastic and non-neoplastic cell lines and tissues. It

can be assumed that TGF-β will be found in conditioned media as well, once the proper assays are used.

Those TGFs which do not compete with EGF for receptor binding and which do not require EGF for colony formation are designated TGF-γ (gamma-type TGF). Such TGFs have been described in conditioned media of certain virally or chemically transformed cells. Finally, TGF-δ (delta-type TGF) is used to specify those TGFs which would both compete for EGF receptors and require EGF for colony formation in soft agar. EGF itself could be classified as a weak TGF-δ.

Example Of Purification And Properties

Research in our laboratory has been directed toward the isolation of TGFs directly from cells and tissues. An acid/ethanol extraction procedure was modified for this purpose, as disclosed in A. B. Roberts, et al., Proc. Natl. Acad. Sci., U.S.A., 77:3494-3498 (1980), and chromatography and high pressure liquid chromatography (HPLC) have been employed for further purification. TGF activity, measured by the ability to induce non-neoplastic indicator cells (NRK) to form colonies in soft agar, has been quantitated on an image analysis system with respect to both the number and size of the colonies formed. By use of HPLC, we have shown that two distinctly different TGFs, here classified as TGF-α and TGF-β, can be isolated from the same pool of acid/ethanol extracts of MSV-transformed 3T3 cells; for this purpose, columns

using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid have been used. TGF-α, which elutes from the column earlier than marker EGF, is characterized by its ability to induce the formation of small colonies (850-3,100 μm^2) in soft agar in the absence of added EGF and its ability to compete with EGF in a radio-receptor assay. TGF-β, which elutes later than TGF-α or marker EGF, does not compete with EGF for receptor binding and requires EGF to induce the formation of large colonies (>3,100 μm^2) in the soft agar assay.

TGF-α from MSV-transformed 3T3 cells resembles SGF isolated from the conditioned medium of the same cells and other TGFs isolated from rat and human tumor cell lines. Recently, SGF and the TGF-α's from the conditioned media of a human melanoma cell line and virally-transformed rat embryo fibroblasts have been purified to homogeneity. The human melanoma TGF-α is a single chain polypeptide of molecular weight 7,400. Its amino acid composition and chromatographic behavior are markedly different from that of human EGF, but similar to that of murine SGF and rat TGF-α, suggesting that TGF-α's from human, rat and mouse genomes are more closely related to each other than to EGF. There is therefore a reasonable possibility that TGF-α's may have cross-species utility.

In sarcoma virus-transformed rodent cell lines, the release of TGF-α into the medium has been correlated with the expression of the transformed phenotype, and within a selected set of human tumor cell lines that release TGF-α, the ability of the tumor cells to grow in soft agar has been correlated with the quantity of TGF-α they secrete. However, secretion of TGF-α is not an absolute requirement for neoplastic behavior; certain

chemically transformed murine cell lines and human lung carcinoma cell lines that do not secrete TGF-α release strong TGF activity that does not compete with EGF for receptor binding.

TGF-β of the acid/ethanol extract of the MSV-transformed 3T3 cells resembles other TGFs isolated from many neoplastic and non-neoplastic tissues. After further purification on a second HPLC column, TGF-β of the MSV-transformed cells eluted at the same position in the n-propanol gradient (48%) as one peak of TGF-β activity of the bovine salivary gland, and each was associated with a small peak of absorbance at 280 nm. These two TGF-β's, one from a neoplastic mouse cell line and the other from a non-neoplastic bovine tissue, each migrated as a 12,500-13,000 daltons MW protein on SDS-PAGE in the presence of mercaptoethanol and as an apparent 25,000-26,000 daltons protein in the absence of mercaptoethanol; they therefore appear to be closely related to each other and different from both TGF-α and EGF. The finding of TGF-β in all non-neoplastic tissues examined thus far suggests a normal physiological function for these TGFs. There is therefore a reasonable possibility that TGF-β's may have cross-species utility.

Amino Acid Composition and Sequencing of TGF-β

Through a combination of techniques, TGF-β from bovine kidneys was purified 200,000-fold to the point of homogeniety.

Samples of homogenous TGF-β (20-50 pmol) were taken to dryness and hydrolyzed in sealed, evacuated tubes at 150°C for 2 hours in 100 µl constant boiling HCl containing 0.1% liquid phenol. Half-cystine and methionine were determined by performic acid oxidation followed by acid hydrolysis. Analyses of o-phthalaldehyde derivatives of the amino acids were done on a modified amino acid analyzer equipped with a fluorometer and computing integrator.

For amino-terminal sequence analysis, approximately 500 picomoles (M_r 25,000) of TGF-β were reduced and S-carboxymethylated with dithiothreitol and iodo-[^{14}C] acetic acid in the presence of 6M guanidine-HCl in 1 M Tris-HCl buffer, pH 8.4. Excess reagents were separated from carboxymethylated protein by HPLC on a 5 micron 50 x 4.6 mm column eluted with a gradient of 0-90% acetonitrile (1% per min) in 0.1% TFA. Overall recovery of the procedure was 96%, based on estimating the amount of protein by amino acid analysis using fluorescamine detection.

Automated Edman degradation was performed on about 500 pmoles (M_r 12,500) of the S-carboxymethylated protein with a gas-phase sequencer. PTH-amino acids were identified using an HPLC system. Initial yeild was about 30% and repetitive yield about 90%.

The following table summarizes the amino acid distribution.

TABLE 1

Amino Acid Composition of Bovine Kidney TGF-β [a]

Amino Acid	Residues/mole[b] (mean ± range)
Aspartic acid	25 ± 1
Threonine	7 ± 1
Serine	16 ± 2
Glutamic acid	26 ± 1
Proline	N.D.
Glycine	15 ± 3
Alanine	18 ± 1
Half-cystine[c]	16 ± 2
Valine	17 ± 1
Methionine[c]	3 ± 0
Isoleucine	11 ± 1
Leucine	25 ± 1
Tyrosine	17 ± 2
Phenylalanine	8 ± 1
Histidine	8 ± 1
Lysine	22 ± 2
Tryptophan	N.D.
Arginine	12 ± 1

[a]Amino acid composition of purified bovine kidney type β TGF determined after 2 h hydrolysis in 6 N HCl at 150°. Values are based on 3 separate determinations of 3 different preparations.

[b]The number of residues per mole for each amino acid is based on an apparent molecular weight of 25,000.

[c]Determined by performic acid oxidation and acid hydrolysis.

N.D. - not determined

Analysis of the bovine kidney TGF-β by electrophoresis on $NaDodSO_4$-polyacrylamide gels suggests that some of the disulfide bonds are interchain. Comparison of the above results with those obtained by the analysis of TGF-β from human placenta or human platelets revealed no significant differences in the respective amino acid compositions, and it is anticipated that the N-terminal sequences will be very similar.

Amino acid sequence analysis of the reduced and S-carboxymethylated bovine kidney TGF-β by automated Edman degradation using a gas-phase sequencer revealed a single N-terminal amino acid sequence as follows, (CMC is S-carboxymethylcysteine):

Ala-Leu-Asp-Thr-Asn-Tyr-CMC-Phe-Ser-Ser-Thr-Glu-Lys-Asn-CMC-.

Initial and repetitive yields were found to be equal to the yields calculated for myoglobin used as standard protein. At the minimum, the results indicate that the sequence of at least the first fifteen N-terminal amino acids of each of the two subunits of TGF-β is identical and confirm the observations of a single protein band of the reduced TGF-β on $NaDodSO_4$-polyacrylamide gels. In addition, the N-terminal sequence of the bovine kidney TGF-β is identical to the partial sequence of TGF-β from human placenta, suggesting a high degree of relatedness of type β TGFs from different species and different tissue sources.

Activation of TGF-β

Recent experiments in our laboratory have shown that either TGF-α or EGF will activate TGF-β to induce the formation of large colonies in soft agar. Purified

TGF-β from the MSV-transformed 3T3 cells, assayed by itself, had no colony-forming activity at concentrations as high as 2 µg/ml. However, assayed after activation by the presence of either EGF, or TGF-α derived from the same cells, TGF-β induced a dose-dependent formation of large colonies (>3,100 μm^2) at concentrations of 10-200 ng/ml. By contrast, EGF or TGF-α, assayed by themselves, induced a maximal response of only a small number of colonies; this response was increased 10-fold by the addition of TGF-β. The relative abilities of EGF and TGF-α to promote TGF-β-dependent formation of large colonies in soft agar correlated with their relative abilities to compete for binding to the EGF receptor; other experiments using chemically-modified EGF analogues have substantiated this correlation. These data, demonstrating that the induction of a strong colony-forming response requires both TGF-α and TGF-β or EGF, suggest that TGF-β, which is found in all tissues, may be an essential mediator of the effects of TGF-α and of EGF on neoplastic transformation.

Little is known about the mechanisms by which exogenous TGFs induce non-neoplastic cells to express the transformed phenotype. Furthermore, the synergistic interactions of TGF-α and TGF-β suggest that these two TGFs may act through different pathways. Experiments using TGFs of conditioned media of sarcoma virus-transformed rodent cells have shown that the synthesis of new RNA and protein is required before transformation occurs. Other experiments have been directed at a possible role of TGFs in phosphorylation reactions. Certain viral transforming gene products and their normal cellular homologues have tyrosine-specific protein kinase activity, and it has been proposed that phosphorylation at tyrosine of specific substrates may be important in the transformation process. Treatment of

human carcinoma A431 cells with various TGFs derived from conditioned media of virally-transformed cells or human tumor cell lines (TGF-α) resulted in phosphorylation of tyrosine residues in the 160 K EGF receptor. The pattern of phosphorylation, however, was indistinguishable from that induced by EGF itself, and thus would not appear to be transformation-specific. Likewise, dissolution of actin fibers of Rat-1 cells occurs when they are treated with either TGF or EGF. It is clear that further research is needed to establish the relationships of the TGFs to the retrovirus transforming gene products and the mode of action of the TGFs in neoplastic transformation.

The following are summaries of examples which illustrate various aspects of this invention:

Example 1.

HPLC separation of TGF-α and TGF-β of MSV-transformed 3T3 cells. The acid/ethanol extract of MSV-transformed cells was chromatographed on Bio-Gel P-30 in 1 M acetic acid. The 7-10,000 MW TGF fraction was further purified on a μBondapak C18 column using a gradient of acetonitrile in 0.1% trifluoroacetic acid. Aliquots were assayed for colony-forming activity in the soft agar assay; in the presence of 2 ng/ml EGF; and in competition with ^{125}I-EGF in a radio-receptor assay.

Example 2.

HPLC purification on μBondapak CN columns of TGF-β from MSV-transformed mouse 3T3 cells and bovine salivary gland using a gradient of n-propanol in 0.1% trifluoroacetic acid. TGF-β of acid/ethanol extracts was purified on Bio-Gel P-30 and μBondapak C18 columns and then applied to CN columns. Aliquots were assayed for induction of colony growth of NRK cells in soft agar in the presence of 2 ng/ml EGF.

Example 3.

Synergistic interaction (activation) of TGF-β with TGF-α to induce the formation of large colonies of NRK cells in soft agar. Soft agar colony-forming activity of varying concentrations of μBondapak CN-purified TGF-β derived from MSV-transformed 3T3 cells was assayed either alone or in the presence of either CN-purified TGF-α derived from the same cells or murine EGF. Soft agar colony-forming activity of varying concentrations of EGF or TGF-α was assayed either alone or in the presence of TGF-β.

In Vivo Demonstration Of Wound Healing

After the above in vitro demonstrations of the operability of the compositions of this invention, it was considered critical to confirm that the compositions could work in clinical applications. For this purpose, TGFs were isolated on a relatively large scale from bovine sources and the wound healing activity of the compositions according to this invention were satisfactorily demonstrated using an experimental rodent wound healing protocol.

The examples which follow demonstrate not only that the compositions according to this invention are effective in vivo, but also that TGFs may be employed cross-species.

Example 4.

Purification and separation of TGF-α and TGF-β. Bovine tissues, obtained fresh from the slaughterhouse and frozen immediately on dry ice, were extracted in 2 kg batches with acid/ethanol in accordance with A. B. Roberts, et al., Proc. Natl. Acad. Sci., U.S.A., 77:3494 (1980). Extracts from 6-8 kg tissue were combined and chromatographed on Bio-Gel P-30 with 1 M acetic acid, using an 80 liter bed volume column. The TGFs of extracts of bovine kidney or bovine salivary gland eluted in a broad peak between the RNase (13,700) and insulin (5,700) markers, as had been observed for the TGFs of mouse kidney and mouse salivary gland. TGFs at this stage of purification had a specific activity approximately 10 to 25-fold higher than the acid/ethanol extracts, with a range of recovery of 150,000-200,000 colony-forming units per kg tissue. Most of the in vivo studies reported below were done with salivary gland or kidney TGFS purified to this stage. The TGFs activity in vitro was enhanced approximately 20-fold by the presence of 2-5 ng EGF per ml in the asssay, in accordance with this invention.

Following chromatography on Bio-Gel P-30, the bovine TGF-β were purified further by High Pressure Liquid Chromatography (HPLC) on μBondapak C18 columns using an acetonitrile gradient in 0.1 percent trifluoroacetic acid, followed by a second HPLC step on μBondapak CN columns using a gradient of n-propanol in 0.1 percent trifluoroacetic acid. After the two HPLC steps, analysis of the bovine TGF-βs from both salivary gland and kidney by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions showed a single band with an apparent molecular weight of 13,000 daltons. At this stage of purification, each of the bovine TGF-β's had an absolute requirement for EGF for colony-forming activity. The yield of HPLC-purified TGF-β was approximately 20-100 μg per kg tissue, with a total activity of 7,000-18,000 colony-forming units.

Example 5.

Wound healing protocol. In vivo activity of isolated salivary gland TGF-β and kidney TGF-β was measured in accordance with the protocol described by T. K. Hunt, et al., Amer. J. Surgery, 114:302 (1967). Six empty Schilling-Hunt wire mesh wound chambers were surgically inserted subcutaneously in the backs of rats, in paired symmetrical fashion (A-D, B-E, C-F) as shown below:

TABLE 2

head

A	D
B	E
C	F

tail

The rats respond to these chambers as if they were wounds, and eventually the chambers become filled with fibroblasts and collagen. By the fourth day after insertion, the chambers become encapsulated with connective tissue, but there are few cells within the chambers themselves. There is thus a defined, enclosed space within the chambers, where a wound healing response can be quantitatively measured. At this time, daily injections of TGF-β (0.1 ml, in sterile phosphate-buffered saline)into chambers A, B, and C were begun. To activate TGF-β activity, a low level of murine EGF was included in all TGF-β injections, unless noted otherwise. Chambers D, E, and F were used as controls, and were injected with either an amount of bovine serum albumin (BSA) alone or in combination with either TGF-β or EGF, such that the total protein was equivalent to the amount of TGF-β injected into chambers A, B, and C. Injections were made once daily for either 5 days (Table 2) or 9 days (Table 3). All injected materials were sterile. The rats were sacrificed 6 hours after the last TGF-β injection; in Table 3 they were injected with 0.5 mCi of thymidine-^{3}H, specific activity 6.7 Ci/millimole (i.p.) together with the last TGF-β injection. The chambers were removed from the rats, all connective tissue on the outside of the wire mesh was peeled away, and then the contents of each chamber were determined.

Table 3. Wound healing response to bovine salivary gland or kidney TGF after 5 days of treatment. TGF-βs were prepared and injected as described. Each dose contained 25 times the amount of TGF-β found optimal for colony formation by NRK cells in a standard soft agar assay, and ranged from 18-42 colony forming units per dose. The amounts of protein injected per dose were: 7 µg in Expts. 1, 4 and 5; 25 µg in Expt. 3, and 0.7 µg in Expt 2. All doses of EGF were 20 ng. Total protein in wound chambers was measured by the method of Lowry, et al.** Statistical analysis of the data was made by comparison of matched pairs of the chambers (A vs. D, B vs. E, C vs. F) shown in Table 2.

Expt.	Chamber A,B,C treatment	Chamber D,E,F treatment	Number of matched pairs of chambers	Milligrams of protein per chamber, average (15) A,B,C	D,E,F	Average ratio ± standard error of mean*	P†
1	TGF-β (Salivary P-30) + EGF	BSA	36	10	3.9	3.8 ± 0.6	<0.001
2	TGF-β (Salivary HPLC) + EGF	BSA	9	8.4	2.9	4.6 ± 1.0	<0.02
3	TGF-β (Kidney P-30) + EGF	BSA	9	8.1	3.5	5.2 ± 1.5	<0.005
4	TGF-β (Salivary P-30) + EGF	EGF	9	9.6	5.3	2.1 ± 0.3	<0.02
5	TGF-β (Salivary P-30) + EGF	TGF-β	9	11.2	9.6	1.4 ± 0.3	0.5

*Average of each matched pair ratio, A/D, B/E, C/F

**J. Biol. Chem, 193:265 (1951)

†One sided P values based on the sign test

Table 4. Wound healing response to bovine salivary gland TGF-β after 9 days of treatment. Chambers A, B, and C were dosed once daily with 7 μg of TGF-β (P-30) plus 20 ng of EGF. Chambers D, E and F were dosed with an equal amount of BSA.

Measurement	Number of matched pairs of chambers	Average content per chamber A,B,C	Average content per chamber D,E,F	Average ratio ± standard error of mean*	P†
Protein, milligrams	30	24	15	1.6 ± 0.05	<0.001
DNA, micrograms	30	21	8.6	2.6 ± 0.16	<0.001
Thymidine-^{3}H, cpm per microgram of DNA	30	45	30	1.7 ± 0.09	<0.001
Collagen, milligrams	9	5.2	3.2	1.8 ± 0.2	<0.005

*Average of each matched ratio pair, A/D, B/E, C/F

†One sided P values based on the sign test

Table 2 shows that 5 days of treatment of rats with TGF-β from either bovine salivary gland or bovine kidney caused a significant increase in total protein in the treated chambers, as compared to control chambers treated with an equivalent amount of bovine serum albumin (Experiments 1, 3). The salivary gland TGF-β was still highly active after two steps of purification by the high pressure liquid chromatography (Experiment 2). The effects observed are not the sole result of the minute amounts of EGF which had been used to potentiate the activity of TGF-β, since a highly significant difference between treated chambers A, B and C, compared to control chambers D, E and F was still observed when EGF was used as the control substance (Experiment 4). Furthermore, when all chambers were treated with TGF-β, and only A, B, and C were treated with EGF, no significant difference was observed (Experiment 5). At the end of Experiments 1-4, it was consistently observed that chambers A, B and C were more firmly fixed in the surrounding connective tissue than the respective matched control chambers, suggesting that effects of the TGF-β also were manifested in the area immediately surrounding the chambers.

In order to measure the effects of bovine salivary TGF-β on DNA and collagen content of the chambers, it was necessary to treat the animals for longer than 5 days. Table 3 shows the results of a larger experiment in which 13 rats were treated for 9 days. The increases in total protein, total DNA, thymidine incorporation into DNA, and total collagen were all highly sufficient. Histological examination of the contents of the chambers treated with TGF-β confirmed the occurrence of fibroblastic proliferation and formation of collagen. A

sterile infiltrate of inflammatory cells was also found within both treated and control chambers.

The results obtained in both experiments indicate that TGF-βs when activated in accordance with this invention, can significantly accelerate a wound healing response.

Clinical Use Of The Compositions Of This Invention

The compositions of this invention, whose active ingredients are TGF-β activated by at least one of a TGF-α and an EGF, can reasonably be expected to have clinical use in the treatment of animals, particularly mammals, most particularly human beings. There are several sound bases for this conclusion.

It has been shown above, that in in vitro tests, the compositions can markedly increase the growth of cells without changing their genotype. An important characteristic of the components of the compositions of this invention, is that they do not appear to be species specific. That is, TGF-β from one species can be activated by TGF-α and/or EGF from other species. The cells whose growth is promoted can be of any type such as fibroblast or

epithelial, although it is considered that the growth promotion of fibroblast cells will have the greatest medical utility.

The in vivo experimental protocol disclosed above, with its very favorable results, clearly indicates that the compositions of this invention have utility in the treatment of traumata by the rapid promotion of the proliferation of the cells surrounding the traumata.

Two types of application of the compositions of this invention are contemplated.

The first, and preferred, application is topically for the promotion of surface wound healing. There are no limitations as to the type of wound or other traumata that can be treated, and these include (but are not limited to): first, second and third degree burns (especially second and third degree); surgical incisions, including those of cosmetic surgery; wounds, including lacerations, incisions, and penetrations; and surface ulcers including decubital (bed-sores), diabetic, dental, haemophiliac, and varicose. Although the primary concern is the healing of major wounds by fibroblast cell regeneration, it is contemplated that the compositions may also be useful for minor wounds, and for cosmetic regeneration of cells such as epithelial. It is also contemplated that the compositions may be utilized by the topical application to internal surgical incisions.

When applied topically, the compositions may be combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be pharmaceutically acceptable, efficacious for their

intended administration, and cannot degrade the activity of the active ingredients of the compositions. When the compositions of this invention are applied to burns, they may be in the form of an irrigant, preferably in combination with physiological saline solution. The compositions can also be in the form of ointments or suspensions, preferably in combination with purified collagen. The compositions also may be impregnated into transdermal patches, plasters, and bandages, preferably in a liquid or semi-liquid form.

The second application is systemically for the healing of internal wounds and similar traumata. Such an application is useful provided that there are no, or limited, undesirable side-effects, such as the stimulation of neoplastic cellular growth.

When applied systemically, the compositions may be formulated as liquids, pills, tablets, lozonges, or the like, for enteral administration, or in liquid form for parenteral injection. The active ingredients may be combined with other ingredients such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be pharmaceutically acceptable, efficacious for their intended administration, and cannot degrade the activity of the active ingredients of the compositions.

The amount of activating agent (TGF-αs or EGFs) present depends directly upon the amount of TGF-βs present in the activated compositions of this invention. There are indications that the activation is not catalytic in nature, and that therefore approximately stoichiometric (equimolar) quantities are preferred.

The amount of activated composition to be used in the methods of this invention cannot be stated because of the nature of the activity of TGFs and the nature of healing wounds and/or other traumata. As indicated above, the TGFs activate cells by binding to receptor sites on the cells, after which the TGFs are absorbed and utilized by the cells for the synthesis of new protein, resulting in cell multiplication. Thus, the TGFs are consumed by the cell regenerating process itself, rather than acting in an enzymatic or other catalytic manner. Receptors for EGFs have been found on a wide variety of fibroblastic, epithelial, and parietal cells, as disclosed in Gonzalez, et al., J. Cell. Biol., 88:108-144 (1981). Further, it has been calculated that there are 3,000 EGF binding (receptor) sites for each rat intestinal epithelial cell, as disclosed in M. E. Lafitte, et al., FEBS Lett., 114(2):243-246 (1980). It must also be obvious that the amount of a cell growth promoting substance (such as the compositions of this invention) that must be utilized will vary with the size of the wound or other traumata to be treated.

Since the compositions of this invention both provoke and sustain cellular regeneration, a continual application or periodic reapplication of the compositions in indicated.

The amount of active ingredient per unit volume of combined medication for administration is also very difficult to specify, because it depends upon the amount of active ingredients that are afforded directly to the regenerating cells of the wound or other traumata situs. However, it can generally be stated that the TGF-βs should preferably be present in an amount of at least about 1.0 nanogram per milliliter of combined composition, more preferably in an amount up to about 1.0 milligram per milliliter.

Additional Embodiments Utilizing the Compositions of this Invention

In addition to utilizing the activated TGF-β compositions of this invention by themselves, it is possible to use them in combination with secondary growth factors.

The activated transforming growth factors of this invention may be physically admixed with one or more of many other (secondary) peptide and non-peptide growth factors. Such admixtures may be administered in the same manner and for the same purposes as the activated transforming growth factors of this invention utilized alone, to enhance their activity in promoting cell proliferation and repair.

The useful proportions of activated transforming growth factor to secondary growth factors are 1:.1-10 mols, with about equimolar amounts being preferred.

The secondary growth factors may be used alone or in any physiologically and pharmaceutically compatible combination.

The known secondary growth factors, in approximately descending order of usefulness in this invention (by group), include:

1. platelet-derived growth factors

2. fibroblast growth factors
   angiogenesis factors

3. insulin-like growth factors including somatomedins

4. insulin
nerve growth factors

5. anabolic steroids.

In addition to the above known secondary growth factors, it is reasonable to expect that as yet undiscovered secondary growth factors will be useful in admixture.

This invention also incorporates the inactive intermediate substance TGF-β per se. Prior to this invention, this substance had not been isolated or identified. TGF-β is believed to be substantially the same or very similar for each animal species, regardless of the individual of that species or the particular body cells from which it is derived. Since TGF-β has been shown to be non-species-specific between rodents, cattle, and human beings, it is also reasonable to believe that the substance is substantially the same or very similar when derived from any mammal, and possibly from any animal source. It should be noted, moreover, that this invention includes TGF-β regardless of the source from which it is isolated or derived, including genetically engineered cells. It is well within the capabilities of biochemical technology to genetically engineer a cell to produce TGF-β at the present time.

Administration of Unactivated TGF-β

It is believed that TGF-β has no wound-healing or other tissue-repair activity unless it has been activated by an agent as described above.

However, it is noted that Table 3 Experiment 5, supra, appears to indicate statistically similar results for TGF-β activated with EGF (chambers A, B, C) and ~~TGF-β~~ per se (chambers D, E, F). The most logical explanation for this, is that the TGF-β per se was activated by a TGF already present in the test animal. Various TGFs, such as EGF, are known to be present in blood plasma.

Thus, the results of Experiment 5 are not inconsistent with this invention, but instead constitute a variant embodiment thereof. Specifically, TGF-β per se may be administered, in accordance with this invention, instead of activated TGF-β, when there are sufficient endogenous activating agents present in an animal, to activate an amount of TGF-β sufficient to promote cell proliferation and tissue repair. It is anticipated that in an animal suffering from the traumata contemplated herein, there usually will not be sufficient endogenous activating agents present.

The disclosures of the following applications, which were filed on the same date as the present continuation-in-part application, are entirely incorporated herein by reference:

1. "Transforming Growth Factor-beta From Human Platelets", by Richard K. Assoian, Charles A. Frolik, Michael B. Sporn, and Anita B. Roberts.
2. "Transforming Growth Factor-beta From Human Placentas", by Charles A. Frolik, Richard K. Assoian, Michael B. Sporn, and Anita B. Roberts.

WHAT IS CLAIMED IS:

1. A composition for the promotion of cell proliferation and tissue repair in animals whose active ingredients consist essentially of:

- beta-type transforming growth factors (TGF-β); and

- at least one activating agent present in an amount sufficient to activate said TGF-β, selected from the group consisting of epidermal growth factors (EGF) and alpha-type transforming growth factors (TGF-α),

- which active ingredients are present in an amount sufficient to promote cell proliferation.

2. The composition of Claim 1, used for the topical promotion of fibroblast cell proliferation in mammals wherein the TGF-β and at least one activating agent are present in approximately equimolar amounts.

3. The composition of Claim 2, wherein said TGF-β is present in an amount of at least 1.0 nanogram per milliliter of composition.

4. The composition of Claim 3, wherein said TGF-β is present in an amount up to 1 milligram per milliliter of composition.

5. The composition of Claim 2, wherein the activating agent is an EGF.

6. The composition of Claim 2, wherein the activating agent is a TGF-α.

7. The composition of Claim 2 further containing at least one pharmaceutically acceptable carrier.

8. The composition of Claim 7, wherein the carrier is a purified collagen and the composition is a suspension for topical application.

9. The composition of Claim 7, wherein the carrier is a physiological saline solution and the composition is an irrigant.

10. The composition of Claim 1, used for the systemic promotion of cell proliferation and tissue repair in mammals wherein the TGF-β and at least one activating agent are present in approximately equimolar amounts and wherein the composition further contains a pharmaceutically acceptable carrier.

11. A method of treating mammalian topical wounds, burns, ulcers or other traumata comprising at least one topical application of the composition of Claim 2 in a pharmaceutically acceptable carrier.

12. The method of Claim 11, further comprising periodic topical reapplication.

13. The method of Claim 11 further comprising continuing topical application.

14. A method of treating mammalian systemic traumata comprising at least one systemic administration of the composition of Claim 10 in a pharmaceutically acceptable carrier.

15. The method of Claim 14, further comprising periodic systemic readministration.

16. The method of Claim 14, further comprising continual systemic administration.

17. The composition of claim 1 wherein the activated TGF-β is admixed with at least one secondary growth factor in a mole ratio of 1:.1-10.

18. The composition of claim 17 wherein the secondary growth factor is at least one the group consisting of: platelet-derived growth factors, fibroblast growth factors, angiogenesis growth factors, insulin-like growth factors, insulin, nerve growth factors, and anabolic steroids.

19. The composition of claim 17 wherein the secondary growth factor is at least one of the group consisting of platelet-derived growth factors, fibroblast growth factors and angiogenesis factors.

20. The composition of claim 18 wherein the activated TGF-β and secondary growth factors are present in about equimolar amounts.

21. The method of claim 11 wherein the activated TGF-β is admixed with at least one secondary growth factor in a mole ratio of 1:.1-10.

22. The method of claim 21 wherein the secondary growth factor is at least one of the group consisting of: platelet-derived growth factors, fibroblast growth factors, angiogenesis growth factors, insulin-like growth factors, insulin, nerve growth factors, and anabolic steroids.

23. Beta-type transforming growth factor.

24. The growth factor of claim 23 derived from a mammalian source.

25. The growth factor of claim 23 derived from a genetically engineered cell.

26. The growth factor of claim 24 derived from a bovine kidney and purified.

27. The growth factor of claim 24 derived from a human source.

28. A transforming growth factor having an amino acid composition consisting essentially of the following residues per mole based upon an apparent molecular weight of 25,000: (wherein ND means not determined)

Aspartic acid	25 ± 1
Threonine	7 ± 1
Serine	16 ± 2
Glutamic acid	26 ± 1
Proline	N.D.
Glycine	15 ± 3
Alanine	18 ± 1
Half-cystine	16 ± 2
Valine	17 ± 1
Methionine	3 ± 0
Isoleucine	11 ± 1
Leucine	25 ± 1
Tyrosine	17 ± 2
Phenylalanine	8 ± 1
Histidine	8 ± 1
Lysine	22 ± 2
Tryptophan	N.D.
Arginine	12 ± 1

29. A transforming growth factor having a partial amino acid sequence as determined by Edman Degredation consisting essentially of the following for each of its two subunits:

Ala-Leu-Asp-Thr-Asn-Tyr-CMC-Phe-Ser-Ser-Thr-Glu-Lys-Asn-CMC-,

where CMC is Half-cystine or cysteine, determined as S-carboxymethylcysteine.

30. A method of treating mammalian topical wounds, burns, ulcers or other traumata comprising at least one topical application of the composition of Claim 23 in a pharmaceutically acceptable carrier.

31. A method of treating mammalian systemic traumata comprising at least one systemic administration of the composition of Claim 23 in a pharmaceutically acceptable carrier.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US83/01460

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

424/177

IPC [3] A61K 37/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U. S.	260/112R, 424/177, Dig.13

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Chemical Abstracts 9th and 10th Collective Index Under "Animal Growth Substances", "Wound" and "Epidermal Growth Factors"

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	Roberts et al., Proc. Natl. Acad. Sci. U.S.A. Vol. 78, No. 9, pp 5339-5343, September 1981	1-16
Y	DeLarco et al., J. Cellular Physiology 109: 143-152, 1981 (p.150)	5

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
23 December 1983	30 DEC 1983
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Donald B. Moyer

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

Invention I: Claims 1-16, compositions and corresponding methods of use.
Invention II: Claims 17-22, a mixture of growth factors.
Invention III: Claims 23-29, compound
Invention IV: Claims 30-31; process of using invention II.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-16

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.